University of Alberta

Effects of nitrogen, pH, and mycorrhizal fungi on the growth, water relations, and physiology of trembling aspen (*Populus tremuloides*) and balsam poplar (*Populus balsamifera*)

by

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ABSTRACT

This dissertation consists of four studies aimed at examining: 1) trembling aspen's response to inorganic nitrogen; 2) how ectomycorrhizal associations may influence trembling aspen's growth and response to inorganic nitrogen; 3) the ectomycorrhizal influence on aspen's response to short-term pH changes, and; 4) how mycorrhizal associations affect growth and water relations of balsam poplar. In the first study, effects of nitrate and ammonium on solution culture-grown trembling aspen were examined. High ammonium concentrations were toxic to aspen, which was not due to pH effects alone. Effects of high ammonium exposure were partly reversible. Although aspen exhibited increased growth with high concentrations of nitrate vs. ammonium, aspen had slightly more growth at low and moderate ammonium concentrations vs. nitrate, suggesting that aspen is capable of moderate ammonium tolerance. In the second study, effects of nitrate and ammonium were examined in sand-grown aspen associated with *Hebeloma crustuliniforme* (Hc). Aspen showed minimal preference for nitrate vs. ammonium, although aspen growth was reduced when nitrate and ammonium were supplied together. N form preference was not related to pH effects. Mycorrhizal aspen had increased nitrogen uptake and ammonium assimilation at higher nitrogen concentrations, but had higher mortality at low nitrogen concentrations. Hc may therefore be important for aspen's increased tolerance of ammonium. In the third study, short-term effects of pH (4-9) on Hc-associated aspen seedlings were examined. Mycorrhizal aspen showed an increased alkaline pH tolerance with increased L_{pr} at pH 7, which was not likely due to changes in AQP activity.. By comparison, L_{pr} and AQP of non-mycorrhizal seedlings were relatively unaffected by pH. In the fourth study, the effects of Hc and *Wilcoxina mikolae* var. *mikolae* (Wm) on balsam poplar growth and water relations were examined. Wm increased balsam poplar's stomatal conductance, shoot water potential, and root volume, but Hc increased balsam poplar's L_{pr}. The different effects of the two fungi on balsam poplar may have been due to the different characteristics of the two fungal species or different types of associations that poplar roots formed with Wm (ectendomycorrhizal) and Hc (ectomycorrhizal).

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CHAPTER I

General Introduction and Literature Review

1.1. Mycorrhizal associations

The symbiotic relationship between mycorrhizal fungi and higher plants has likely evolved as a survival mechanism for the purpose of tolerating and mitigating the effects of environmental stresses. More than 90% of the world's plants have mycorrhizal associations (Trappe 1987, Smith and Read 1997), with arbuscular (AM) and vesicular-arbuscular mycorrhiza (VAM) comprising most of these associations (Smith and Read 1997). In tree species, mycorrhizal associations are primarily ectomycorrhizal (Brundrett et al. 1996), but some genera such as *Pinus, Picea,* and *Larix* are also known to exhibit ectendomycorrhizal associations (Peterson et al. 2004). Other genera such as *Acacia*, *Cupressus*, *Juniperus*, *Tilia*, *Ulmus*, and *Eucalyptus* are known to exhibit VAM or AM associations in addition to ectomycorrhizal associations, depending on soil conditions, presence of inocula, and age of the tree (Smith and Read 1997).

1.1.1. Types of associations

There are seven different types of mycorrhizal associations. They are: ectomycorrhiza (ECM); endomycorrhiza, now known as vesicular-arbuscular (VAM) or arbuscular mycorrhiza (AM); ectendomycorrhiza; ericoid; orchid; arbutoid; and monotropoid (Mukerji et al. 2000). Both plant and fungal species show specificity in the symbiont species with which they form associations. Some plant species have a wide range of fungal associations, other plant species have a narrow range, and the same is true for fungal species (Molina et al. 1992b, Peterson et al. 2004). Interspecies compatibility is determined by both plant (Walker et al. 1986) and fungal (Kropp and Fortin 1987) genetics.

1.1.2. Ectomycorrhizal fungi

Characteristic structures of an ectomycorrhizal (ECM) association are the mantle which forms a hyphal sheath surrounding parts of the root, with additional hyphal projections that extend into the rhizosphere, and the Hartig net which forms a hyphal matrix that surrounds individual epidermal and cortical cells in the root (Peterson et al. 2004). Fungal penetration of the root by the Hartig net is restricted to the epidermal cells in angiosperms, forming a paraepidermal Hartig net, whereas in gymnosperms the Hartig net may encompass the epidermis and cortical cells (Peterson and Massicotte 2004). Hyphae do not penetrate cell protoplasts, with the apoplastic interface between fungus and plant occurring at the junction of fungal cell walls and adjacent plant cell walls, respectively (Peterson and Massicotte 2004). ECM fungi only infect secondary and tertiary roots, often resulting in the production of short, dichotomous root branching in coniferous tree species with certain fungal species (Kaska et al. 1999) and in the reduction or disappearance of root hairs in infected roots (Ditengou et al. 2000). ECM fungi can also result in extensive root branching (Karabaghli-Degron et al. 1998, Tranvan et al. 2000), and both the branching pattern and mantle patterns can be used in fungal morphotyping identification (Brundrett et al. 1996).

ECM fungi are associated with fewer tree species than VAM or AM fungi, but are ecologically significant because they are associated with 140 tree genera belonging to the Myrtaceae, Mimosaceae, Pinaceae, Fagaceae, Salicaceae, Betulaceae, and Juglandaceae families (Brundrett et al. 1996), and because these types of associations dominate temperate, boreal, and sub-tropical forest ecosystems (Alexander 1989). ECM fungi are almost exclusively basidiomycetes and ascomycetes (Mukerji et al. 2000). No monocotyledonous species are known to have ECM associations (Mukerji et al. 2000).

Hyphal penetration of roots is achieved mechanically or by fungal excretion of pectinases (Mukerji et al. 2000, Peterson and Massicotte 2004). Several metabolic (Smith and Read 1997) and developmental (Kottke et al. 1997) changes, and alteration in gene expression (Duplessis et al. 2005, Le Quéré et al. 2005, Morel et al. 2005, Frettinger et al. 2007) in both plant hosts and ECM fungi occur at the start of symbiosis, with possible changes prior to physical contact (Duchesne 1989). Roots possess proteins associated with ECM roots called ectomycorrhizins (Hilbert et al. 1991) that may be involved in forming an association. Fungi may also produce (Karabaghli et al. 1998, Neimi et al. 2000) or alter the production of hormones such as auxins, or work in conjunction with these plant-produced hormones to form an ECM association (Neimi et al. 2002). The key event in symbiosis is hyphal adherance to plant root cells; this requires both cellular differentiation and changes to the structure and composition of cell walls, and regulation of cell wall proteins may also be involved (Tagu and Martin 1996). However, the series of signaling mechanisms that begin the process of creating a symbiotic association and the conditions required to initiate formation of symbiosis are not known.

1.1.2.1. *Hebeloma crustuliniforme*

Hebeloma crustuliniforme (Bull.) Quél. (order Agaricales) is a basidiomycete with a wide distribution in North America, Europe, and other parts of the world, and is often found in or near open wooded or heathland areas (Phillips 1991). It is common in the boreal forest and has a wide range of plant hosts (Kernaghan and Currah 1998). It is capable of forming ECM associations with several gymnosperm and angiosperm tree species, especially members of the Salicaceae family such as the *Populus* and *Salix* genera (Aanen et al. 2000a), as well as with species of *Betula*, *Tilia* (Aanen et al. 2000b), *Quercus* (Lunt and Hedger 2003), *Pinus* (Rosling et al. 2004), and *Picea* (Brunner et al. 1991, Muhsin and Zwiazek 2002b). Since it is an early-colonizing species, synthesized ECM associations using *H. crustuliniforme* with young tree seedlings can be readily compared to naturally-occurring symbioses (Brunner et al. 1991).

1.1.3. Ectendomycorrhizal fungi

Ectendomycorrhizal (EEM) associations have structural characteristics that are intermediate between both ECM and endomycorrhizal fungal structures. EEM associations are defined by these structures and also by the fungal species involved (Trevor et al. 2001). These fungi form a Hartig net that can penetrate through all layers of the root cortex (Scales and Peterson 1991a). They may also form a mantle, which may be thin or nonexistent, and produce intracellular hyphae found within cortical cells (Trevor et al. 2001). The functionality of the apoplastic interface between plant and EEM fungal symbiont has not been studied, although sites of interaction include the interface between plant cells and fungal hyphal cells as is the case for ECM symbioses, and the development of a perifungal membrane surrounding the intracellular hyphae (Peterson and Massicotte 2004), although the characteristics and composition of the perifungal membrane has not been studied (Scales and Peterson 1991a). The intracellular hyphae may be involved in nutrient exchange with the plant host as it is with endomycorrhizal associations, or it may be a latent pathogenic stage in senescing roots when nutrient absorption from tree roots by the fungi would occur (Trevor et al. 2001). EEM associations in angiosperms that have been characterized solely by the presence of intracellular hyphae may have been senescent ECM associations (Högberg 1982, Louis 1988).

EEM fungi are known to have a wide range of plant hosts (Molina et al. 1992a,b), a broad global distribution, and a broad habitat range, and likely have significance in the growth and survival of many tree species (Massicotte et al. 1999). Ecologically, EEM associations have been observed over a broad pH range, in disturbed environments, in nurseries, and on sites of low productivity, and can utilize multiple sources of simple sugars; they may be important for revegetation and re-establishment of conifer seedlings following disturbance (Danielson 1991, Trevor et al. 2001). Not much is known about the tolerance of EEM fungi to heavy metals, pollution, and high temperatures (Trevor et al. 2001), but peak growth rates around 20°C have been reported, with no growth at or below 4°C except in northern isolates (Wilcox et al. 1983). However, the ecological and physiological roles with respect to broadleaf species are unknown. Although early research reported EEM associations in broadleaf species (Laiho 1965), the findings were questionable due to characterization of field-collected roots by researchers (Trevor et al. 2001).

Some species of EEM fungi are known as E-strain fungi because they have the ability to form EEM associations with some plant species and ECM associations with other plant species (Trevor et al. 2001). Most isolates of E-strain fungi belong to the *Wilcoxina* genus, particularly *W. mikolae* and *W. rehmii* (Egger et al. 1991). *Sphaerosporella brunnea* is another E-strain fungus capable of forming ECM with many species of *Picea*, *Pinus* and *Larix*, as well as *Populus tremuloides* (Danielson 1984).

Wilcoxina mikolae var. *mikolae* is also capable of forming ECM associations with *Picea mariana* and *Betula alleghaniensis* (Scales and Peterson 1991b).

1.1.3.1 *Wilcoxina mikolae*

Wilcoxina mikolae (Yang & Korf) Yang & Korf and *W. mikolae* var. *mikolae* (Yang & Wilcox) Yang & Korf, (order Pezizales) is an ascocarp that is considered an E-strain fungus. *Wilcoxina mikolae* and *W. rehmii* are genetically and ecologically similar, and are commonly associated with conifers from the *Pinus* (Ivory and Pearce 1991), *Picea*, and *Larix* genera (reviewed in Trevor et al. 2001, Peterson et al. 2004). In particular, *Wilcoxina* spp. has been found and studied in tree nurseries, and may be the most abundant mycorrhizal species present in nurseries, especially if organic fertilizer is used (Trocha et al. 2006). Ecologically, it is found in areas following disturbance (Trevor et al. 2001), such as fire (Fujimura et al. 2005), due to its ability to withstand a disturbance or to recolonize a disturbed area (Smith et al. 2005). Not much is known about the natural ecology or distribution of *Wilcoxina* spp., because most research involving this genus has occurred in nurseries or with synthesized associations. However, it has been found in forested areas (Hart et al. 2005, Smith et al. 2005), and is presumed to have a wide distribution and to be associated with conifers in many regions of the world. Although *Wilcoxina* spp. has not been studied ecologically or physiologically in association with angiosperms, it is possible that *Wilcoxina* may form ECM or EEM associations with angiosperms if introduced (Peterson et al. 2004).

Wilcoxina mikolae and *W. rehmii* are unique in that, like some algal species, they produce ferricrocin, an iron-chelating siderophore that solubilizes and improves iron uptake under low-iron conditions; ferricrocin may also form stable complexes with other metals, providing protection to tree hosts exposed to toxic levels of heavy metals by binding heavy metals in a non-reactive manner (Prabhu et al. 1996a). It may be possible to use ferricrocin-producing mycorrhizae in remediation of metal-contaminated soil.

1.1.4. Effects of mycorrhizal associations

In tree species, mycorrhizae are thought to be important for tree survival in temperate and boreal forests (Read and Perez-Moreno 2003) in the defense against several biological stresses, including drought (Davies et al. 1996, Morte et al. 2001, Muhsin and Zwiazek 2002a), pH extremes (Kernaghan et al. 2002, Wallander 2002), high (Izzo et al. 2006) and low (Landhäusser et al. 2002, Tibbett et al. 2002) soil temperatures, heavy metal toxicity (Colpaert and Van Assche 1987, Jones and Hutchinson 1988, Jentschke and Godbold 2000), salinity (Dixon et al. 1994, Kernaghan et al. 2002), transplant stress and winter survival (Smith and Read 1997), the presence of toxins (Marx and Artman 1979, Gardner and Malajczuk 1988, Peiffer and Bloss 1988), nutrient deficiency (Plassard et al. 2000, Van Tichelen and Colpaert 2000), root diseases, and plant pathogens (Strobel and Sinclair 1991, Barea et al. 1998, Sylvia and Chellemi 2001). Fungal symbionts provide increased root hydraulic conductivity (Bogeat-Triboulot et al. 2004, Marjanović et al. 2005a) due to both increased water flux through cell membranes (Muhsin and Zwiazek 2002a) and also through the apoplast (Landhäusser et al. 2002). They improve nutrient absorption (Smith and Read 1997), and mineral (N, P, K, Ca, Zn) absorption, particularly of phosphorous (Bolan 1991, McElhinney and Mitchell 1995, Van Tichelen and Colpaert 2000). Increased absorption is accomplished through increased root surface area and translocation to plant tissues (Mukerji et al. 2000). Improved plant nutrition can result in higher net assimilation rates (Loewe et al. 2000), higher photosynthetic rates, higher nutrient content of tissues (Lehto 1992), and increased growth and yield (Smith and Read 1997, Muhsin and Zwiazek 2002b). Fungi receive carbohydrates from their plant hosts. They may also form common mycorrhizal networks between two or more plant species, distributing or redistributing assimilated nutrients, especially carbon, between plant hosts of the same or different species, and thereby influencing plant interactions and interspecies competition (reviewed in Simard and Durall 2004). In forest soils, mycorrhizae also significantly contribute to nutrient cycling and organic matter turnover by breaking down more complex mineral and organic matter in the F and H horizons of the forest floor, thereby making existing soil nutrients more readily available to plants (Mukerji et al. 2000).

The relationship between mycorrhizae and host plants has previously been considered a classic mutualistic symbiosis due to many observed benefits derived from plants and fungi. This theoretical mutualism is currently under review due to several previous observations that included no apparent effect of mycorrhizae on plant hosts, reduced plant growth and nutrient uptake at the whole-plant and cellular levels, and increased mortality and necrosis in mycorrhizal plants. Effects have ranged from no derived plant benefits to parasitic effects of mycorrhizae in plants (reviewed in Jones and Smith 2004).

1.1.5. Mechanisms of the benefits of mycorrhizal associations

The particular mechanisms by which mycorrhizal fungi confer benefits to plant hosts under stress conditions are poorly understood. Evidence of nutrients moving from fungus to plant (Hodge 2003) and carbon compounds being moved from mantle and Hartig net to roots (Scales and Peterson 1991a) has been reported. Fungi, with their extended hyphal projections into the soil from the mantle, may greatly increase the root surface area so that plants can exploit more soil volume to acquire water and nutrients (Duddridge et al. 1980), as well as increasing root systems' water transport capacity (Muhsin and Zwiazek 2002a, Bogeat-Troboulot et al. 2004). Mycorrhizal fungi also have been shown to help deter root infection by soil-borne fungal pathogens through the use of chemical (Garrido et al. 1982) and physical barriers (Duchesne et al. 1989), and also by the production of metabolites in soil and plants that increase plant resistance to infection (Zeng 2006). However, in EEM fungi, the mantle probably offers no barrier to pathogens or to apoplastic movement of nutrients from soil to root (Trevor et al. 2001). Mycorrhizae provide growth hormones (auxins, gibberellins, cytokinins) and growth regulators (B vitamins) to the host plant (Ho 1987, Kraigher et al. 1991, Gopinathan and Raman 1992) that may be involved in changes in physiological processes that will benefit the plant host. There may also be molecular changes occurring in the plant and fungal cell walls as part of the formation of symbiosis (Duplessis et al. 2002, Tagu et al. 2002), as well as cytochemical changes involving phosphate (Chalot et al. 2002) and sugar (Nehls et al. 2001, Chalot et al. 2002) transporters, enzymes (Dexheimer et al. 1986), and H^+-ATPases

(Lei and Dexheimer 1988) occurring in plant and fungal cells that facilitate transfer of nutrients.

1.1.6. Factors affecting mycorrhizal associations

The outcome of a given symbiotic association is difficult to predict, and likely varies with the environmental conditions affecting both plant and fungal symbionts (Johnson et al. 1997, Zhou and Sharik 1997). Although mycorrhizal associations have generally been characterized as mutualisms, a new categorization of mycorrhizal associations has arisen in which an association is classified as "balanced" or "exploitative", depending on whether or not the dynamic exchange of essential materials required for growth and survival between symbionts is bidirectional or one-sided (Brundrett 2004). Alternatively, a continuum ranging from mutualism to parasitism has been proposed to describe the variability in mycorrhizal symbioses (Bronstein 1994, Johnson et al. 1997)

Although many benefits to host plants from mycorrhizal associations have been reported, there have also been many reports of negative or no effects on growth or nutrient acquisition in mycorrhizal plants; several biotic and abiotic factors have been proposed as being confounding factors that were not taken into account but that could have explained the perceived negative effects of mycorrhizal fungi (reviewed in Jones and Smith 2004). Some of these factors include genetic variability of plant and fungus, the presence of soil bacteria and invertebrates that may impact growth and function of plants or fungi, the great difficulty of establishing true nonmycorrhizal controls both in pot and field studies, long-term or delayed benefits that are not measurable in a short-term experiment, and benefits at the cellular level that are not evident at the whole-plant level (Jones and Smith 2004). Additionally, edaphic and geographic variability can be confounding factors (Mukerji et al. 2000).

Wilcoxina mikolae has been shown in one experiment to lower nitrogen content of leaves and shoot biomass, but when *Bacillus polymyxa* was introduced to root systems, *Pinus contorta* did not suffer any negative effects, showing that bacterial-fungal interactions can vary the results of mycorrhizal benefits (Chanway and Holl 1991), especially if the bacterium is capable of fixing atmospheric nitrogen (Smith and Read 1997).

Another factor affecting mycorrhizal associations is the ability of the fungal species to produce its own carbohydrates. It has been suggested that, due to their ability to break down complex polysaccharides in soil (Caldwell et al. 2000), EEM fungi are able to colonize roots during early plant developmental stages, which occurs frequently in nurseries. This strategy allows EEM fungi to acquire sufficient carbohydrates for their own needs without draining the carbon resources of young seedlings. ECM species, by comparision, may require substantial carbon for development (Mikola 1988). However, the cost of EEM associations in young seedlings has not been studied (Trevor et al. 2001).

The extent and diversity of mycorrhizal associations can change with time, both with respect to root maturity and rate of root growth (Smith and Read 1997, Khasa et al. 2002). Other factors, such as competition between fungal species, and environmental conditions including changes associated with clearcutting (Dahlberg and Stenström 1991), nitrogen fertilization (Kårén and Nylund 1997), and liming (Andersson and Söderström 1995), may also alter the types and extent of mycorrhizal communities, as well as their associations with plant species.

1.2. Root water relations

1.2.1. Root anatomy

All roots of woody plants have a similar structure, consisting of an epidermal layer surrounding the outside of the root, a cortex which may be comprised of several cell layers, the number of which is dependent on species and developmental stage of the root, and a vascular cylinder at the center, consisting of xylem, phloem, and ringed with an endodermis (reviewed in Steudle and Peterson 1998). The endodermis is a layer of cells surrounded by a Casparian band, comprised of lignin which is hydrophilic, and suberin which is hydrophobic (Zeier and Schreiber 1998). The endodermis can restrict the movement of water between cells when it is moving from the cortex to the vascular tissue (Melchior and Steudle 1993, Zimmerman et al. 2000), but may not act as a complete barrier to apoplastic water flux (Peterson et al. 1981, Steudle et al. 1993). In some species, depending on growing conditions, there is also a layer of exodermis just below the epidermis, which is similar in composition to the endodermis (Hose et al. 2001). The properties of the endodermis and exodermis may result in water and dissolved solutes being forced to pass from the cortex to the vascular tissue through the cell membranes, due to the presence of suberin lamellae which can block apoplastic flux (Sanderson 1983, Steudle and Peterson 1998). However, the presence of passage cells that can mediate the passage of water and solutes in both the endodermis and exodermis has been reported (Peterson and Enstone 1996).

1.2.2. Root water absorption and movement

Water and nutrients entering the plant must enter through the root system (Henzler et al. 1999), traveling through the apoplastic or cell-to-cell pathways before entering the vascular tissue (Steudle 1994, Steudle and Peterson 1998). Although the apoplastic pathway is that of least hydraulic resistance due to the absence of membranes (Steudle et al. 1993), the suberization of cell walls in woody root tissues, particularly in the exodermis and endodermis, may limit apoplastic water flux (Tyerman et al. 1999). The cell-to-cell pathway, therefore, is considered to be the dominant pathway for water flux in most, if not all root systems, although it is extensively mediated by the presence of transmembrane protein water channels called aquaporins (Johansson et al. 1998, Voicu and Zwiazek 2004, Aroca et al. 2006). The proportion of apoplastic vs. cell-to-cell water flux can change due to changes in membrane permeability (Steudle and Peterson 1998) and also due to changes in root age and environmental conditions, including drought stress (Taleisnik et al. 1999, Siemens and Zwiazek 2004), osmotic stress (Carvajal et al. 1999, Engels 1999), and mycorrhizal associations (Coleman et al. 1990, Muhsin and Zwiazek 2002a,b), which may either enhance or restrict water movement based on anatomical and physiological changes in root systems (Cruz et al. 1992, Peterson et al. 1999). It has also been suggested that plants may be able to selectively regulate apoplastic flux with long-term changes in the structural anatomy of the exodermis (Hose et al. 2001)

1.2.2.1. Role of aquaporins

Aquaporins (AQPs) are a large family of transmembrane protein water channels with highly-conserved amino acid sequences in plants, animals, and fungi, capable of passively transporting molecules of water (Maurel and Chrispeels 2001, Siefritz et al. 2002) and small uncharged solutes through plant cell membranes (Uehlein et al. 2003). They are regulated by gene expression (Barrieu et al. 1998, Lopez et al. 2003) via hormones (Kaldenhoff et al. 1993, Phillips and Huttly 1994) and by activation and deactivation via protein kinases and phosphatases that induce a conformational change to restrict water movement through the channel (Johansson et al. 1998). Changes in AQP activation and gene expression can be initiated by environmental signals and stresses such as blue light (Kaldenhoff et al. 1993), diurnal patterns of water transport (Lopez et al. 2003), drought (Yamaguchi-Shinozaki et al. 1992, Morillon and Lassalles 2002), salinity (Azaizeh et al. 1992), and temperature (Lee et al. 2005, Rhee et al. 2007). AQPs are involved in both short-term and long-term regulation of water flux at the cellular level (reviewed in Javot and Maurel 2002). Water movement through AQPs can also be partially and reversibly inhibited by the addition of mercurial compounds that bind to sulfhydryl groups of cysteine amino acid residues located near the surface of the outer cell membrane (Eckert et al. 1999, Lovisolo and Schubert 2006).

Mycorrhizal fungi may influence AQP expression or activity in plant roots (Uehlein et al. 2007) in response to drought (Marjanović et al. 2005b) and salinity (Ouziad et al. 2006). AQPs may also be up- or down-regulated in the mycorrhizal fungal tissue (Frettinger et al. 2007), but there is very little research on aquaporin regulation in mycorrhizal fungi.

Nitrogen nutrition is also important for root water relations, as nitrogen deficiency has been known to increase both radial and axial hydraulic resistances of roots to water flux to the point where AQP-mediated radial water flux was non-responsive to $HgCl_2$ (Li and Shao 2003). Metaxylem vessels were also found to be smaller as a result of nitrogen deficiency (Li and Shao 2003). The form of nitrogen is important in water relations, as supplied nitrate can result in greater root hydraulic conductivity than when ammonium is supplied, and this is not due to increased root mass or root suberization (Adler et al. 1996, Guo et al. 2002). It is theorized that effects of nitrogen on root water relations involve H^+, Ca^{2+}, and K^+ signaling (Netting 2000, Tazawa et al. 2001), and possibly nitrogen metabolism (Barthes et al. 1996).

1.2.2.2. Methods for studying apoplastic water flux

Based on studies of root anatomy and differential resistances to root water flux, and on the current knowledge of AQPs, it has been theorized that the percentage of apoplastic flux through roots must be relatively small. Previous research has attempted to empirically validate this assumption with the use of fluorescent tracer dyes to quantify apoplastic flux (reviewed in Steudle and Peterson 1998). These studies have yielded variable results and variable success, depending upon the dye used, the applied stress conditions, and the presence of any root damage. Although 1-2% apoplastic flow was generally reported in these studies, a maximum of 52% apoplastic flow was found in disturbed roots (Hanson et al. 1985, Moon et al. 1986, Yeo et al. 1987, Skinner and Radin 1994). The main argument for use of apoplastic tracer dyes is that they are restricted to the apoplastic pathway because of their molecular size, but this has also been one of the arguments against their use for apoplastic quantification, because their rate of transport through the apoplast may differ from that of water (Hanson et al. 1985, Yeo et al. 1987). Sulforhodamine G had been used to indicate entry-points where water may enter into the symplast from the apoplast (Varney et al. 1993). Rhodamine B (RB) (Wan and Zwiazek 1999) and trisodium 3-hydroxy-5,8,10-pyrenetrisulfonic acid (PTS_3) (Siemens and Zwiazek 2003, 2004) have been used to quantify changes in apoplastic flux in root systems. These and other tracer dyes, such as rhodamine G (RG), 1,3,6,8-pyrenetetrasulfonic acid (PTS_4), lucifer yellow CH dipotassium salt (LY), and 4-acetoamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), were comparatively tested for their efficacy to measure apoplastic flux and for their potential toxicity to plants by Skinner and Radin (1994). There were large differences between the different dyes when their relative concentrations in xylem exudate were used as estimates of apoplastic flux (Hanson et al. 1985, Moon et al. 1986, Yeo et al. 1987, Skinner and Radin 1994). However, PTS_3 and PTS_4 were found to be the least likely to interfere with transpiring plants and root water flux, and were less likely to accumulate in cell walls, both of which are disadvantages of using RB and RG (Gurr 1971, Skinner and Radin 1994). SITS was found to affect leaf water potential, and the cost of LY for large-scale use were some disadvantages of using these other tracer dyes (Skinner and Radin 1994). However, because variable results have been obtained from different tracer dyes, and because plant and environmental factors may affect apoplastic flux measurements, it is now considered more appropriate to interpret changes in dye-based apoplastic flux measurements as relative rather than absolute differences (Wan and Zwiazek 1999, Siemens and Zwiazek 2004).

1.2.2.2.1. PTS$_3$

PTS$_3$ is a fluorescent tracer dye that is restricted to movement through the apoplastic pathway in roots (Skinner and Radin 1994). The dye has previously been used in water relations studies to quantitatively measure apoplastic flow because it does not adhere to cell walls or accumulate in tissues (Peterson et al. 1981, Moon et al. 1986, Skinner and Radin 1994). While it is now known that studies using PTS$_3$ cannot be used as an absolute measure of apoplastic flow due to the dye molecule's size and negative charge (Zimmerman and Steudle 1998), it has been useful in studies where relative changes of apoplastic flow have been measured in response to changes in an environmental or applied stress condition (Siemens and Zwiazek 2003, 2004).

1.3. Nitrogen in forest soils and plants

Nitrogen is a component of many organic compounds such as amino and nucleic acids, chlorophyll, and enzymes. As a macronutrient, plants require considerable amounts of nitrogen for optimal physiological function. In many forest ecosystems, nitrogen and phosphorous are the most limiting nutrients.

1.3.1. Forest soils

Nitrogen, along with phosphorus, is often the most limiting nutrient for plants within forests (Tilman 1986, Tamm 1991, Vitousek and Howarth 1991) because it is the most abundant element found in plant tissues after carbon, hydrogen, and oxygen (Kozlowski and Pallardy 1997). Overall, there is more total nitrogen per unit volume in deciduous forest soils than coniferous forest soils (Côté et al. 2000, Jerabkova et al. 2006). This is because N content of deciduous leaf litter is higher than that of coniferous leaf litter, and because decomposition rates of deciduous leaf litter are generally higher than those of coniferous leaf litter, there is higher nitrogen turnover in deciduous forests (Kozlowski and Pallardy 1997). In undisturbed mature boreal forest soils, ammonium is often the dominant form of inorganic nitrogen (Blew and Parkinson 1993, Prieto-Fernandez et al. 1993). In well-aerated temperate soils, nitrate can dominate due to bacterial conversion of organic matter to nitrate which, as an anion, can move freely through soil due to its high solubility and its negative charge which prevents its binding to the cation-exchange-complex (Nye and Tinker 1977). Nitrate has also been found in acidic forest soils (Vogt and Edmonds 1982). Several factors affect the quantity and form of nitrogen present and available to plants, including plant species composition (Eviner and Chapin 1997, Kronzucker et al. 1997) and forest disturbances that can increase the nitrate concentration in temperate and boreal forest soils such as fire, clearcutting, and windthrow (Kronzucker et al. 1995a,b).

Nitrate concentrations in mature undisturbed coniferous forests tend to be low relative to ammonium concentrations (Gosz and White 1986, Kronzucker et al. 2003), Ammonium:nitrate ratios can change with different forest types and environmental conditions (Nadelhoffer et al. 1985), with lower ratios in previously disturbed areas (Walley et al. 1996). In undisturbed forest soils, the ammonium:nitrate ratio may be 10:1 (Carlyle 1986). The ammonium:nitrate ratio was found to be higher in deciduous (33:1) than in coniferous (5:1) boreal forests (Jerabkova et al. 2006). Typical ammonium concentrations in soil vary widely depending on soil type, region, season, soil water content, soil horizon, and environmental site factors. Ranges of 2.4 – 12.5 mg kg^{-1} ammonium and <0.1 – 2.8 mg kg^{-1} nitrate within mineral soils over a range of deciduous forest soils, and 1.4 – 12 mg kg^{-1} ammonium and 0 – 4.5 mg kg^{-1} nitrate over a range of coniferous forest soils, have been reported (Vitousek et al. 1982). In two boreal forest studies, deciduous soils had 3.57 mg kg^{-1} ammonium and 0.11 mg kg^{-1} nitrate, and coniferous soils had 2.23 mg kg^{-1} ammonium and 0.44 mg kg^{-1} nitrate, with some mixed forest soils having intermediate values (Schmidt et al. 1996, Jerabkova et al. 2006). Ammonium and nitrate concentrations are higher in the organic forest floor layers where seedling establishment occurs, compared with the underlying mineral layers (Balisky et al. 1995, Jerabkova et al. 2006).

1.3.2. Nitrogen cycling in soils

Major biological processes that are part of nitrogen cycling include: nitrogen fixation, the bacterial conversion of atmospheric nitrogen to ammonium; ammonification, the decomposition of nitrogenous organic matter to ammonium; nitrification, the bacterial oxidation of ammonium to nitrite and then to nitrate; nitrogen assimilation of either ammonium or nitrate into complex molecules by plants; and denitrification, the bacterial conversion of nitrate to atmospheric nitrogen (Schlesinger 1997).

Nitrogen cycling plays an important role in making nitrogen available to plants. In addition to having higher total nitrogen availability (Myrold et al. 1989, Bormann and Sidle 1990, Ste-Marie and Paré 1999), deciduous forests also have higher nutrient turnover than coniferous forests (Vogt et al. 1986, Pastor 1987). This difference may be non-existent in older stands (Paré and Bergeron 1996).

Ammonium and nitrate, either fixed from atmospheric nitrogen or mineralized from the breakdown of organic matter, may be used as a source of nitrogen by plants (Epstein and Bloom 2005). Many soil microbes seem to prefer organic over inorganic sources of nitrogen (Chapin et al. 1993), and ammonium over nitrate, as sources of nitrogen for assimilation (Jansson et al. 1955). Nitrification and nitrate assimilation by microbes is important, and is affected by the C:N ratio in soil. Large additions of carbon to forest soils, as well as C accumulation in undisturbed forest soils, increases the C:N ratio and will result in lower rates of nitrification; this may also partly explain low nitrate concentrations in undisturbed forest soils (Hart et al. 1994, Walley et al. 1996). However, spatial variation in soils may result in heterogeneous nitrate availability, with pockets of high rates of nitrification relative to the rest of the soil (Stark and Hart 1997). Fungal assimilation and transportation of nitrate from areas where mineralization is occurring to where carbon is highly available may increase nitrate assimilation rates in localized areas (Stark and Hart 1997).

Forest succession and tree species diversity in a given ecosystem are partly determined by changes in nitrogen chemistry of soil (Eviner and Chapin 1997, Kronzucker et al. 1997). Given that many tree species require nitrogen in the form of nitrate (Britto and Kronzucker 2002), their existence in mature, undisturbed forest soils where low levels of nitrate persist has not been explained (Eyre 1980, Alban 1982, Kronzucker et al. 2003). The low levels of nitrate that have been typically observed in mature forest soils and related measurements of microbial nitrification and nitrate assimilation have been shown to be inconsistent with each other, since reported soil nitrate levels have been much lower than measured nitrification rates suggest (Stark & Hart 1997). This may be due to underestimation of actual gross nitrification rates via net nitrification rate measurements (Davidson et al. 1992), or because soil microbes are capable of assimilating nearly all soil nitrate in undisturbed forest ecosystems at rates much higher than nitrate assimilation by plants (Stark & Hart 1997). Nitrification may be important in some undisturbed conifer forests, generating significant amounts of nitrate that are immediately assimilated by both bacteria and plants, keeping soil nitrate levels low (Walley et al. 1996, Stark and Hart 1997), although other findings suggest that nitrification rates were not significantly different between decidous and coniferous forests (Paré and Bergeron 1996, Jerabkova et al. 2006). In trees, it has been shown that available soil N may be more affected by the N content of litterfall than by litter decomposition rates (Vitousek et al. 1982, Nadelhoffer et al. 1983, Jerabkova et al. 2006), but it also suggests that nitrogen may not be as limiting to plants as previously thought, or that plants are not as competitive for nitrogen as are soil microbes (Gholz 1982, Vitousek et al. 1982, Stark and Hart 1997). Low levels of nitrification could be due to lower populations of nitrifying bacteria (Blew and Parkinson 1993), low soil pH, or nitrifying inhibitors present in soil (Vitousek and Matson 1985).

Forest soil disturbances influence nitrogen cycling and may result in nutrient losses such as: ion leaching due to clearcutting (Vitousek et al. 1982); mineral volatilization from fires (Raison et al. 1985); and increased nitrification from increases in soil pH and bacterial composition (Kutiel and Naveh 1987). Nitrate and carbon tend to accumulate in disturbed areas, with increases in the C:N ratio (Walley et al. 1996). It has been suggested that aspen that rapidly reestablish on disturbed sites can act as a nitrogen sink, limiting nitrate accumulation in soil (Walley et al. 1996). Although periodic disturbances, such as fire, in the boreal forest explains how nitrogen-demanding species such as aspen become readily established in disturbed areas (Kronzucker et al. 2003), it does not explain their continued survival as forest successional changes, and associated changes in soil pH and nitrogen availability, occur.

1.3.3. Importance of nitrogen for plants

The importance of nitrogen as an element for plants is its incorporation into several molecular building blocks needed for the manufacture of cellular components and processes such as photosynthesis, including nucleic acids and nucleotides, amino acids and proteins, polyamines, carbon compounds and other metabolites (Taiz and Zeiger 2002, Epstein and Bloom 2005). As an anion (NO_3^-), it serves as a charge-balancing ion, and an osmoticum in the regulation of osmotic concentrations (Mengel and Kirkby 2001, Taiz and Zeiger 2002, Epstein and Bloom 2005). Nitrogen is a mobile nutrient, and symptoms of nitrogen deficiency include general chlorosis, particularly in lower, older leaves, etiolated habit, slow growth, and a spindly appearance (Epstein and Bloom 2005). After carbon, hydrogen, and oxygen, nitrogen is the most abundant nutrient in plant tissues, with adequate total nitrogen tissue concentrations averaging approximately 1.5% in tissues of most higher plants (Kowlowski and Pallardy 1997). Ranges between 1.1 – 1.7% in shoots, and 0.9 – 1.3% in roots have been reported in *Pinus sylvestris* (Wallander 2002). In mature *Populus tremuloides*, leaves may have over 2% total nitrogen during the growing season (Henry 1973).

1.3.4. Nitrogen metabolism in plants

Inorganic nitrogen in a plant is absorbed through roots as a combination of nitrate or ammonium ions from soil (Williams and Miller 2001). For many plants, ammonium is the preferred form (Bloom 1997), particularly in conifers and shade-tolerant species

(Stewart et al. 1992, Templer and Dawson 2004). This may be due to the prevalence and availability of ammonium in soils in soils compared with other forms of nitrogen (Epstein and Bloom 2005). However, some species such as *Fagus grandifolia*, *Acer saccharum*, and *Quercus rubra* seem to prefer ammonium over nitrate even when growing in temperate nitrate-rich soil with high rates of nitrification (Templer and Dawson 2004). Some plants may also be able to absorb and assimilate organic amino acids as a source of nitrogen, acquiring them directly from soil in arctic or alpine areas where organic forms of nitrogen predominate (Chapin et al. 1993) and from boreal forest soils with and without the assistance of mycorrhizal symbionts (Näsholm et al. 1998, Persson and Näsholm 2001).

1.3.4.1. Ammonium uptake

Much of the nitrogen taken up by plants growing in forest soils is in the form of ammonium (Rennenberg et al. 1998), although nitrate and organic nitrogen can be present in substantial quantities in ammonium-rich forest soil (Rennenberg et al. 1998, Persson and Näsholm 2001). Ammonium uptake in roots is stimulated by rhizospheric ammonium concentrations (Feng et al. 1994) and is regulated by a membrane potential-dependent, ammonium-specific, high-affinity plasma membrane uniport (Kleiner 1981, Glass et al. 1997), which has been genetically-sequenced and found in many plant tissues (Ludewig et al. 2002). The charge of transported ammonium ions is balanced by the ammonium-stimulated generation of protons to lower the pH of the cytoplasm, which stimulates H^+-ATPase pumping of protons out of the cell (Kurkdijan and Guern 1989, Serrano 1989). Influx of ammonium is stimulated by the lower pH outside of the cell to balance the ionic charge (Tyerman 1992). As ammonium is taken up from soil, rhizospheric pH may decrease up to 3 pH units, which further increases ammonium availability (Marshner et al. 1991). Net ammonium uptake by roots is negatively correlated with external nitrogen concentration (Morgan and Jackson 1989, 1998) and in some cases with intracellular glutamine concentration (Lea et al. 1992).

In ECM fungi, ammonium uptake may be mediated by a saturable, proton symport (Botton and Chalot 1995).

1.3.4.2. Nitrate uptake

Nitrate uptake is limited by its availability (Stark and Hart 1997). Nitrate uptake by roots is not passive across the plasma membrane, but occurs against a concentration and a charge gradient via an energetically-driven high-affinity transport system, which is an H^+/NO_3^- symport highly specific for nitrate (Ullrich 1992), as opposed to other similar anions, with optimal absorption at or below pH 6 due to the need for H^+ availability (Vessey et al. 1990). Expression of this symport can be up- or down-regulated by nitrate concentration, depending on the molecular type of nitrate transporter and the plant species (Williams and Miller 2001). The extent of root nitrate absorption can also either be inhibited (Kronzucker et al. 1999) or stimulated (Smart and Bloom 1988) by the presence of non-toxic levels of ammonium, presumably because in some cases the presence of ammonium would alter the pH and availability of H^+, but also because ammonium may affect the activities of enzymes involved in nitrate assimilation (Aslam et al. 1997). Uptake of nitrate can raise the pH of the rhizosphere, decreasing further nitrate uptake (Rygiewicz et al. 1984b).

In some fungal species, nitrate may be directly taken up by nitrate reductase that may be associated with the plasmalemma (Pateman and Kinghorn 1976).

1.3.4.3. Nitrogen assimilation

Assimilation of nitrate and ammonium by plants into amino acids differs. Reported ammonium-induced growth inhibition of some plant species is a contentious subject (Gerendás et al. 1997, Britto and Kronzucker 2002) because ammonium is a prevalent intermediate in the nitrogen metabolism of plants. Ammonium in high concentrations is toxic to plants because the conversion of ammonium to ammonia is a reversible, pH-dependent reaction where $NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O$, with ammonia being able to diffuse across cell membranes; ammonium is therefore either assimilated into amino acids close to the absorption site or stored in vacuoles (Epstein and Bloom 2005). Nitrate accumulation in plant tissues does not result in the same toxic effects, compared with the effects of ammonium accumulation, and can be readily stored and transported between tissues before assimilation into amino acids (Epstein and Bloom 2005).

1.3.4.3.1. Nitrate

Two particular enzymes are involved in the assimilation of nitrate, which is eventually converted to ammonium. The first step is catalyzed by nitrate reductase (NR) in the cytosol, which converts NO_3^- to NO_2^- with either NADH (in root tissues) or NADPH (in root and leaf tissues) (Oaks 1994):

$$NO_3^- + NAD(P)H + H^+ + 2e^- \rightarrow NO_2^- + NAD(P)^+ + H_2O$$

NR is activated and deactivated by dephosphorylation and phosphorylation, respectively, of the enzyme's serine residues, and its expression (Sivasankar and Oaks 1996) and activity (Kaiser and Huber 1994) increase with nitrate concentration, as well as with light and carbohydrate levels. Nitrite is then transported into chloroplasts (in leaves) or plastids (in roots) and converted to ammonium via a redox reaction involving nitrite reductase (Siegel and Wilkerson 1989). Although both roots and shoots can convert nitrate to ammonium, the extent of nitrate assimilation in separate tissues is dependent upon the plant species (Pate 1973, Andrews 1986b) and the concentration of nitrate supplied to plants. Many plants assimilate nitrate in roots when nitrate concentration is low, with a shift to nitrate assimilation in shoots as nitrate concentration increases (Marschner 1995). In general, temperate species assimilate most of the nitrate in roots (Scheromm and Plassard 1988).

Nitrate assimilation in ECM fungi is similar to that in plants, with the exception of the nitrite reductase reaction, which uses NADPH and pyridine nucleotides as electron donors (Plassard et al. 1984).

1.3.4.3.2. Ammonium

Ammonium from root absorption, nitrate assimilation, or photorespiration, is assimilated into amino acids via two possible pathways (Mifflin and Lea 1980, reviewed in Joy 1988). The fact that ammonium assimilation requires less energy than nitrate assimilation (Reisenauer 1978) may be a potential reason for ammonium preference by some plants. The primary pathway, called GS-GOGAT, involves the actions of two sequential enzymes, glutamine synthetase (GS), and glutamate synthase, also known as glutamine-2-oxoglutarate aminotransferase (GOGAT) (Lea et al. 1992). GS catalyzes the initial reaction: Glutamate + NH_4^+ + ATP → Glutamine + ADP + P_i

There are two classes of GS: one is found in cytoplasm and produced in germinating seeds and root or shoot vascular bundles, where it produces glutamine for intracellular transport; the other is found in chloroplasts and root plastids, where expression is altered by light and carbohydrate levels, and it produces amide nitrogen that is used locally in roots or reassimilates photorespiratory ammonium in shoots (Joy 1988, Lam et al. 1996). GOGAT activity is stimulated by high levels of glutamate, and catalyzes the following reaction with either NADH or ferredoxin, assimilating ammonium directly from soil in roots or assimilating photorespiratory ammonium in leaves:

Roots: Glutamine + 2-oxoglutarate + NADH + H^+ → 2 Glutamate + NAD^+

OR

Leaves: Glutamine + 2-oxoglutarate + Fd_{red} → 2 Glutamate + Fd_{ox}

However, particularly under conditions of nitrate nutrition, the Fd-GOGAT type of enzyme assimilates ammonium in root plastids (Epstein and Bloom 2005).

An alternate pathway for ammonium assimilation involves another enzyme, glutamate dehydrogenase (GDH) which catalyzes the following reaction (Stewart et al. 1980): NH_4^+ + 2-oxoglutarate + NAD(P)H + H^+ → glutamate + H_2O

This pathway directly converts ammonium to glutamate in a single step, but is currently thought to play only a small role in total ammonium assimilation in many plant species (Joy 1988, Magalhães et al. 1990). Glutamine and glutamate are then further assimilated into amino acids via transamination reactions catalyzed by aminotransferases such as aspartate aminotransferase (Roberts et al. 1970).

Therefore, GS and GDH are potentially two important enzymes that control the process of ammonium assimilation in plants. Although there is little information on the physiological mechanisms involved in controlling ammonium assimilation (Oaks and Hirel 1985), genetic studies may provide some information (Harrison et al. 2000).

Ammonium assimilation in ECM fungi is different, in that GDH, located in the cytoplasm, may be the primary pathway for ammonium assimilation (Marzluf 1981), and has been found in a few ECM species (Martin et al. 1983, Chalot et al. 1990b, Ahmad and Hellebust 1991). In fungi, there are two forms of GDH: NAD-GDH, which has a high affinity for glutamate and is likely involved glutamate synthesis (Garnier et al. 1997); and NADP-GDH, which has a high affinity for ammonium and is mainly involved in glutamate degradation (Brun et al. 1992). Enzymatic activity in fungi is sometimes maintained while in symbiosis (Botton et al. 1989), but has otherwise been shown to be modified or suppressed by the plant hosts, possibly in part due to mycorrhizal structural changes (Malajczuk et al. 1987, Botton and Dell 1994). Other studies have reported that GS has been found in abundance in ECM fungi (Chalot et al. 1991, Brun et al. 1992) and may be the main pathway for ammonium assimilation due to its high affinity for ammonium compared with GDH (Chalot et al. 1990a, Brun et al. 1992). Because fungi require a particular C:N ratio to assimilate nitrogen, they break down and resynthesize glutamine, recycling nitrogen in order to maintain optimal carbohydrate to nitrogen ratios (Hernandez and Mora 1986).

1.3.4.4. Nitrogen in xylem exudate and location of nitrogen assimilation

The nutritional status and location of inorganic nitrogen assimilation within plants may be assessed with analysis of xylem exudate, since xylem vessels unidirectionally transport water from roots to shoots, which may contain dissolved ions and organic and inorganic compounds absorbed from soil and manufactured in roots. Nitrate may be a major component of xylem exudate because many plants are capable of reducing nitrate in shoots rather than in the roots (Siebrecht and Tischner 1999), although the opposite occurs in many temperate tree species (Scheromm and Plassard 1988). Nitrate is also used to balance ionic charge equivalents in xylem sap (Hoarau et al. 1996). Amino acids may also be present in xylem sap (Miranda et al. 2002), with glutamine being the dominant amino acid in xylem sap if ammonium is assimilated in roots (Sauter 1981, Millard et al. 2006).

Although some research assumes that all inorganic nitrogen is assimilated in the same location, there is evidence that locations for nitrate vs. ammonium assimilation may be different. Research has been conducted with several plant species with respect to determining the location of nitrate and ammonium assimilation patterns, although mostly crop species have been the subject of such study. This previous research has consisted of quantifying relative concentrations of nitrate, ammonium, and amino acids in xylem exduate, as well as ^{15}N uptake studies. For example, tomato (*Lycopersicon esculentum*) assimilated ammonium directly in roots, but nitrate assimilation occured in either roots or shoots (Evans et al. 1996). This was also found to be the case for *Ricinis communis* (Peuke and Jeschke 1993). Stresses such as salinity and drought can reduce nitrate transport to the shoot in plants that tend to assimilate nitrate in leaves, thereby resulting in a shift of nitrate assimilation to the roots (Lips 1997). Mycorrhizal association may also affect relative proportions of nitrate and ammonium assimilated in roots vs. shoots, as was the case in *Lactuca sativa* (Azcon et al. 1992). To date, the author has found little scientific evidence of ammonium transport to shoots from these papers, since many plant species seem to assimilate most ammonium directly at or near the point of uptake.

Concentrations of nitrogen in xylem sap may vary between 9 mM – 20 mM, depending on low or high nitrogen fertilization (Wilkinson et al. 2007), and depending on species. Tree species tend to have much lower nitrate concentrations than crop species (Holzapfel et al. 2002, Kawachi et al. 2002), which can range between <0.01 mM to 8 mM nitrate, and up to 20 mM total nitrogen, depending on nutrient status and diurnal fluctuations (Pate and Jeschke 1993, Siebrecht and Tischner 1999, Siebrecht et al. 2003). Depending on the plant species, experimental design, and environmental conditions, changes in form of nitrogen fertilization may result in changes in the form and quantity of nitrogen found in xylem exudate, or in changes in the concentrations and presence of other ions and organic compounds; such changes may occur within days, but may also occur over a much longer period of time (Peuke at al. 1996, van Beusichem et al. 1998, Siebrecht and Tischner 1999). Although some studies report changes in xylem sap nitrogen composition occurring as expected with changes in supplied nitrate and ammonium, other studies report contradictory findings, as well as unexpected changes with other components such as concentrations of anions (SO_4^{3-}, and Cl^-) and cations (K, Ca, Mg) (Allen and Smith 1986, Arnozis and Findenegg 1986, van Beusichem et al. 1998, Siebrecht and Tischner 1999).

Regardless of whether nitrogen is assimilated in roots or shoots, nitrogen and amino acid concentration in xylem sap have been shown to change in response to changes in the amount and form of supplied nitrogen (Andrews, 1986a,b, Peuke et al. 1996, Lexa and Cheeseman 1997), including nitrate (Gojon et al. 1991). Nitrogen concentrations in plants are also affected by seasonal changes (Sauter 1980), which can result in springtime remobilization of nitrogen in the form of glutamine (Millard et al. 2006). The composition of xylem sap is not uniform, exhibiting concentration and pH gradients along the shoot, in part because of nutrient transfer between xylem and phloem (Berger et al. 1994, Schill et al. 1996).

Changes in nitrogen content within xylem sap can also have an effect on sap pH, which has been observed to be highly variable with unpredictable values due to a number of factors (Gerendás and Schurr 1999, Levy et al. 1999). Xylem pH may fluctuate diurnally and seasonally between 4 – 7 for most species, with the majority of values between 5 – 6.5 (Gerendás and Sattelmacher 2002). Seasonally, it may fluctuate from 4.5 during dormancy in winter to 5.5 – 6.5 during the growing season (Glavac et al. 1990). Although the effects of forms and concentration in xylem sap pH are not understood, it has been shown that reductions in sap nitrogen result in increased sap pH (Kirkby and Armstrong 1980, Gollan et al., 1992, Schurr et al., 1992, Dodd et al. 2003). In other studies, an increased supply of nitrogen to the soil and detached leaves can also lead to increased xylem and apoplastic pH (Mengel et al. 1994, Hoffman and Kosegarten 1995), which does not occur via the mechanism whereby alkalinization of xylem sap occurs under nitrogen deficiency or water deficit (Wilkinson 2004, Wilkinson and Davies 2002). Because the pH of xylem sap affects ion translocation (Wolterbeek 1987) and because an acidic pH is associated with stomatal opening and leaf expansion (Davies et al. 2002, Wilkinson and Davies 2002), the relationship between xylem sap pH and nitrogen status of the plant merits further investigation since there has been little research in this area.

1.3.5. Ammonium sensitivity in early-successional species

Many species of plants exhibit symptoms of ammonium toxicity when exposed to high levels of ammonium (Magalhães et al. 1992, Fangmeier et al. 1994, Gerendás et al. 1997, Kronzucker et al. 1997), including species that are considered ammonium-tolerant (Liao et al. 1994). Trees have adapted to prefer one form of inorganic nitrogen over another, with some tree species being particularly sensitive to the presence of ammonium, even at moderate concentrations (Britto and Kronzucker 2002). Many early-successional plants and crop species perform well on nitrate-rich soils, with long-term ammonium exposure resulting in toxicity and mortality (de Graff et al. 1998, Britto and Konzucker 2002). Examples of early-successional tree species with a preference for nitrate are trembling aspen and Douglas-fir (de Visser & Keltjens 1993, Min et al. 2000), poplars (Pearson and Stewart 1993), western redcedar (Krajina et al. 1973), and Scots pine (Vollbrecht et al. 1989). In contrast, white spruce, a late-successional species, has a widespread distribution and dominates late-successional temperate and boreal forests (Farrar 1995). Based on laboratory research, it has been theorized that white spruce may be excluded from nitrate-rich, disturbed sites due to its inability for uptake and metabolization of nitrate, although this was not verified experimentally in field studies (Kronzucker et al. 1997). Symptoms of ammonium toxicity include severe chlorosis, growth suppression, lowered root:shoot ratios in some species, increased mortality rates, and reduced mycorrhizal associations, lower tissue concentrations of cations (K^+, Mg^{2+}, Ca^{2+}), increases in certain anions (Cl^-, SO_4^{2-}, and PO_4^{3-}), and changes in organic acid concentrations that cannot be entirely attributed to rectification of cytosolic pH alone (reviewed in Britto and Kronzucker 2002). Ammonium toxicity has been observed in experiments using buffered growth media (Bligny et al 1997, Blacquèire et al. 1998).

Determining the underlying physiological mechanisms responsible for ammonium sensitivity and toxicity in some species is a contentious subject. There have been many proposed explanations for ammonium sensitivity which are based on a substantial but often contradictory body of research. Such explanations include: possible acid-intolerance of plant species as a result of excess uptake of cations with ammonium, leading to acidification of tissues and the rhizosphere due to an efflux of protons from roots (Vitousek et al. 1982, van Beusichem et al. 1988, Yan et al. 1992, Schubert and Yan 1997); the low-affinity transport system responsible for ammonium uptake is not downregulated (Cerezeo et al. 2001), acting like a non-selective K^+ channel (White 1996, Kronzucker et al. 2001); deficiency of essential cations due to excess ammonium (Cakmak and Marschner 1992, Gerendás et al. 1997, Britto and Kronzucker 2002), particularly potassium (Wang et al. 1996); excess carbon stores in roots, preventing ammonium from being assimilated due to an unfavourable C:N ratio (Kubin and Melzer 1996, Schortemeyer et al. 1997); and low capacity of the GS-GOGAT pathway (Fangmeier et al. 1994). Other theories are also reviewed and refuted by Britto and Kronzucker (2002).

However, the most plausible theory for ammonium toxicity is that of futile ammonium transmembrane cycling, proposed by Britto et al. (2001b), to explain the excessive accumulation of free ammonium in the cytosol of plant tissues, and excess efflux of ammonium ions from cell membranes in ammonium-sensitive species (Husted et al. 2000, Britto et al. 2001b, Cerezo et al. 2001). Ammonium influx is sufficient for growth (Kronzucker et al. 2003) but is likely passive, unlike the efflux system in ammonium-sensitive plants (van Beusichem et al. 1988, Britto et al. 2001a, Schjoerring et al. 2002). Efflux is energy-dependent and poorly regulated, occuring via a high-capacity transport system that operates against a concentration and a charge gradient, resulting in transport inefficiency and high respiration rates associated with ammonium efflux (Larson and Rees 1994, Britto et al. 2001a). In comparison, ammonium-tolerant species seem to exhibit a cell membrane depolarization that lowers the membrane potential gradient, so that ammonium efflux requires less energy and is conducive to lowering cytosolic ammonium concentrations (Britto et al 2001b).

Net accumulation of ammonium over nitrate in ammonium-sensitive species does not result in a growth advantage, and can inhibit growth (de Visser and Keltjens 1993, Min et al. 1999). It has been shown that both early- and late-successional species have much higher rates of net nitrogen acquisition with ammonium compared with nitrate at very low and typical soil concentrations (Kronzucker et al. 2003). Symptoms of ammonium toxicity have been observed in field studies (Oltshoorn et al. 1991, de Visser and Keltjens 1993). The addition of nitrate to ammonium-rich growth media can alleviate or entirely prevent toxicity in some species, and may result in improved growth that is better than with either source of nitrogen provided alone (Deignan and Lewis 1988, Hecht and Mohr 1990, Cruz et al. 1993, Kronzucker et al. 1997). This may be due to nitrate induction of the GS-GOGAT pathway (Redinbaugh and Campbell 1993). Environmentally, ammonium sensitivity may become a problem due to nitrogen deposition and pollution in the forms of ammonium or ammonia from livestock and agriculture (Pearson and Stewart 1993, Fangmeier et al. 1994).

1.3.6. Influence of mycorrhizae in nitrogen metabolism and uptake

It is thought that mycorrhizal fungi can improve nitrogen uptake and assimilation in plants partly due to their ability to enzymatically break down (Bending and Read 1995) and absorb complex organic sources of nitrogen (Wallander 2002), including humus (Bending and Read 1995), chitin (Hodge et al. 1996), proteins (Finlay et al. 1992), amino acids (Näsholm et al. 1998), and other fungi (Andersson et al. 1997). Mycorrhizal enzymes used for breaking down organic matter consist of extracellular proteases, and they are able to maintain this enzymatic activity even when in association with plant roots (Ramstedt and Söderhäll 1983, El-Badaoui and Botton 1989). *Hebeloma crustuliniforme*, a mineral soil-dwellng ECM fungus, produces an acid protease with an optimal activity within a pH range from 2-5, in response to a complex mix of organic nitrogen sources. This protease is not inhibited by inorganic forms of nitrogen (Zhu et al. 1990). However, there are other species of ECM fungi that inhabit organic soil horizons that are capable of producing several types of proteases (El-Badaoui and Botton 1989). Enzymatic metabolism may therefore be a species-dependent limiting factor in mycorrhizal nitrogen metabolism (Plassard et al. 1991).

ECM fungi have also been shown to take up inorganic nitrogen (nitrate, ammonium) at much higher rates in pure culture, many with a preference for ammonium over nitrate, than when they are in symbiosis with mycorrhizal plants (Botton and Chalot 1995), indicating that the extent of nitrogen uptake may be modified due to plant-fungal interactions (Rygiewicz et al. 1984a), which are not understood.

Although the nitrogen metabolism of E-strain fungi has not been thoroughly studied, it is known that they are able to utilize both nitrate and ammonium (Mikola 1965). *Wilcoxina mikolae* var. *mikolae* has been found to possess an NADPH-specific nitrate reductase that is induced by nitrate and suppressed by ammonium (Prabhu et al. 1996b). Although E-strain fungi cannot metabolize cellulose as a carbon source, they are like other mycorrhizal fungi in their ability to use simple sugars (Mikola 1965), which is important in the maintenance of optimal C:N ratios for nitrogen metabolism.

ECM fungi and their ability to form associations with plant hosts are also affected by high levels of nitrogen, which lessen or inhibit root colonization (Smith and Read 1997). EEM fungal colonization and mantle formation is enhanced by phosphorus and urea fertilization (Fortas and Chevalier 1992, Pachelweski et al. 1992), but inhibited by ammonium (Mikola 1965). Not much is known about the effect of nutrient conditions on EEM fungi (Trevor et al. 2001). Other environmental factors, such as increased rates of nitrogen deposition and conditions that favour nitrification (such as temperate soils and a higher pH), may affect the mycorrhizal community by favouring species of fungi that are more capable of utilizing inorganic sources of nitrogen (Taylor et al. 2000) and nitrate (Read 1991), respectively.

It is also possible that part of the improvement of mycorrhizal uptake of nitrogen and other limiting nutrients such as phosphorus is due to their ability to exploit a greater soil volume via the large surface area provided by their hyphal extensions from roots (Read 1991).

ECM fungi can both positively and negatively affect nitrogen availability and cycling processes in the soil, because many species are known to have either an inhibitory (Olsson et al. 1996, Olsson and Wallander 1998) or a stimulatory (Nurmiaho-Lassila et al. 1997, Olsson and Wallander 1998) effect on the presence and growth of soil bacteria. ECM fungi which stimulate bacterial growth produce organic acids (oxalic, citric), which are a food source for some bacteria (Wallander 2000, Jones et al. 2001).

ECM fungi may be involved in increased ammonium tolerance of early-successional tree species, possibly by increasing ammonium uptake in the fungal tissue, by somehow preventing ammonium from interacting with root cell membranes (Plassard et al. 2000, Constable et al. 2001), via the down-regulation of low- or high-affinity ammonium transporters under conditions of typical ammonium concentrations in forest soils (Kronzucker et al. 2003), or possibly via chemical conversion of ammonium in the fungal tissue to a less toxic form of nitrogen that can be directly transferred from fungus to plant root (Ek et al. 1994, Kronzucker et al. 2003). However, there has been little research in this area.

Mycorrhizal assimilation of nitrate and ammonium is also somewhat different from their plant hosts, partly due to differences in the enzymatic pathways (see 1.3.4.3.). Additionally, the enzymes involved in nitrogen assimilation may have different inducers and inhibitors than plant-based enzymes (Plassard et al. 1986).

1.4. The importance of pH and its effects on plants, fungi, and the environment

pH is important for both plants and the environment, and regulates such aspects as nutrient solubility and mobility in soils and plant tissues, changes in soil structure and decomposition, activity and composition within the soil microbial community, physiological functions in plants, and ion exchange (Rengel 2002). The pH of a typical soil is dependent on the chemical composition (Marschner 1986), disturbance (Walley et al. 1996), influences of industrial practices, pollution, and nitrogen deposition (Goulding et al. 1998) and associated plant species (Epstein and Bloom 2005), with differences in the pH ranges of deciduous (4.0 – 5.6) and coniferous forests (3.1 – 5.0) (Vitousek et al. 1982, Raulund-Rasmussen and Verje 1995, Jerabkova et al. 2006). Commercial and agricultural soils may have a pH range from 6 – 8 (Canadian Council of Ministers of the Environment 1999), whereas a fibric peat soil may have a pH range between 3.6 – 4.2, a mesic peat soil between 5.4 – 7.5 (Macyk and Turchenek 1995), and oilsands-affected areas between 7.6 – 8.5 (Canadian Petroleum Association 1980, Macyk et al. 1992).

1.4.1. Physiological importance of pH in plants

Regulation of intra- and extracellular pH is accomplished via membrane-bound H^+-ATPases, some of which serve to pump protons out of the cytoplast (Jahn et al. 1998). Some ATPases are used to create a proton gradient across cell membranes, which is used to actively transport solutes through membrane carriers, and this gradient is necessary in such diverse physiological processes as stomatal opening, creating a driving force for nutrient uptake, and active nutrient transport within plants (Michelet and Boutry 1995, Palmgren 1998). Some of these carriers are also H^+ symports (Felle 2002). The pH of both the cellular apoplast and the vacuole in plants is approximately 5.5, with fluctuations between 5.1-5.9, whereas the pH of the cytoplasm is approximately 7.2 - 7.4 (Jahn and Palmgren 2002, Epstein and Bloom 2005).

Maintaining a somewhat acidic pH within soil, at the root-soil interface, and also at the cell membrane interface, is necessary for nutrient uptake by enchancing mineral solubility and mobility in soils, since protons are used in cation exchange reactions to displace positively charged minerals (Ca^{2+}, Mg^{2+}, K^+, Mn^{2+}, Fe^{3+}) from negatively charged soil particles (Jahn and Palmgren 2002) and in altering ion concentrations such as NO_3^- in the apoplast (Felle 2002). Under extremely acid conditions, some nutrients may become insoluble (Ca^{2+}, Zn^{2+}, Mg^{2+}, PO_4^{3-}), may be leached from soil (N), or become unavailable (NO_3^-) since nitrification is prevented at low pH, with most N present in the form of NH_4^+ (Larcher 1980). Many soils are considered to have low fertility at low pH due to low concentrations of micronutrients (Marschner 1986). However, other minerals are extremely soluble at low pH (Al, Fe, Mn), and may be present in concentrations toxic to plants (Larcher 1980, Marshner 1986). At high pH, there may be plenty of essential nutrients (Ca, Mg), but boron may be present in concentrations toxic to plants (Marschner 1986).

Within cells, pH is tightly controlled because of the necessity of maintaining a membrane potential, and also because H^+-ATPase activity has a pH optimum of approximately 6.5, which can be shifted upward by altering the lipid composition of the membrane (Palmgren et al. 1988). The pH-dependent activity of H^+-ATPases with different pH optima may play a role in conferring acid tolerance to organisms (Axelsen et al. 1999, Luo et al. 1999). Regulation of H^+-ATPase expression and activity can occur in response to a variety of environmental stimuli (reviewed in Jahn and Palmgren 2002). Mechanisms of posttranslational regulation of H^+-ATPases is under debate, but may be accomplished by phosphorylation and dephosphorylation with protein kinases and phosphatases (Lino et al. 1998, Olsson et al. 1998).

1.4.1.1. Influence of pH on plant nitrogen uptake

Soil and rhizosphere pH influence uptake of nutrients, including nitrogen, as well as root hair initiation (Bibikova et al. 1998), and the ability to regulate intracellular pH through the functioning of H^+-ATPases (Schubert and Yan 1997). Factors affecting soil pH include decomposition of organic matter and microbial processes, acid rain, and excretion of protons and organic acids from roots (Jahn and Palmgren 2002). Factors affecting intracellular pH include chilling stress and excess CO_2 (Maeshima and Nakanishi 2002). Nitrogen uptake differs between root zones, dependent on pH changes within the rhizosphere along the root axis (Fischer et al. 1989, Miller et al. 1991). Increased nitrate and calcium uptake corresponds to an increasing pH up to 7.5 (Rygiewicz et al. 1984b), although nitrate uptake can occur under acid soil conditions when soil phosphorus content is low (Gahoonia et al. 1992). A high or low soil pH will also affect solubility of minerals necessary for plant growth, with acid soils having high concentrations of aluminum, iron, and manganese, and alkaline soils having high concentrations of boron, calcium due to calcareous bedrock (Larcher 1980, Marshner 1986), and sometimes salts such as sodium which may be naturally-occurring or due to anthropogenic influences (Brady and Weil 2002). Nitrogen may be deficient in alkaline soil (Marschner 1986).

Acidic soils are usually associated with low species productivity (Fageria et al. 1997), nutrient deficiencies (P, Ca, Mg, Mo) (Li et al. 1991, Marschner 1991), and poorly-developed microbial communities and fungal associations (Abbott and Robsen 1985, Roberts 1995), although some crops show enhanced productivity at low pH (Clark and Zeto 1996, Mendoza and Borie 1998). Although nitrification is not absent or restricted by low pH, it may be a contributing factor to lower levels of nitrate if the pH is

< 5.8 (Pennington and Ellis 1993, Walley et al. 1996). Liming can enhance nitrogen availability in acid soils (Andersson and Söderström 1995), as can mycorrhizal enhancement of phosphorus uptake (Ortas et al. 2004), and toxicity tolerance (Clark et al. 1999, Rohyadi et al. 2004).

Cytosolic and xylem pH is also influced somewhat by the form of nitrogen supplied, with nitrate resulting in higher proton efflux and higher xylem pH values (5.37) than with ammonium (5.03) (Raven and Smith 1976, Raven 1986), although not all studies demonstrate this effect (Zornoza and Carpena 1992). This efflux from roots can further acidify soil, especially in acid-tolerent species such as hemlock which may exhibit higher proton efflux from roots (Ryan 1983, Rygiewicz et al. 1984a). The extent of nitrate storage within vacuoles also alters vacuolar pH and pH homeostasis between the vacuole and cytosol (Maeshima and Nakanishi 2002).

1.4.2. Significance of pH changes in environment

Different types of soils have different pH optima to which native species are adapted, therefore a change in pH can have either negative or positive consequences for the site ecology and physiology of plants on-site (Rengel 2002). Increased pH due to liming, as used in forestry and agricultural practices to balance a calcium or magnesium deficiency (Hüttl and Zöttl 1993), has been shown to increase fine root biomass and length, resulting in overall improvement in tree growth (Bakker 1999), but has also resulted in fungal species-dependent changes in the percentage of mycorrhizal root tips (Bakker et al. 2000). Higher pH soils may also alter the mycorrhizal community within the soil, favouring mycorrhizal species that prefer organic rather than inorganic forms of nitrogen (Wallander et al. 1997). Oilsands mining can raise the pH (8.8) of soil and mine tailings, as well as increase calcium and sodium concentrations (Li and Fung 1998, Zwiazek et al. 1998), which can result in toxicity and solubility problems as outlined above, as well as lower nitrogen uptake (Wallander 2002). Nitrogen deposition from atmospheric pollution and industry in addition to fertilization has resulted in slight to extreme lowering of pH in soils over the last several decades, as well as changes to nitrogen cycling processes, to the point of decline and disappearance of pH-sensitive plant species (reveiewed in Goulding et al. 1998).

1.4.3. Role of mycorrhizal fungi in pH tolerance and nitrogen uptake

Ectomycorrhizal fungi themselves do not necessarily modify the pH of their environment, but can be affected in their growth and nitrogen uptake by pH (Wallander 2002). Some mycorrhizal species show increased root colonization with increased pH, with an optimal colonization rate between pH 5-7, however any correlation between colonization rates and increased yield or nutrient uptake in plant hosts is fungal species- or isolate-dependent (Medeiros et al. 1994). Mycorrhizal fungi can colonize roots at pH values as low as 2.7 (Ballen and Graham 2002). In ectendomycorrhizae, pH has been shown to change the structural characteristics of fungi that colonize roots, with optimal and typical structures for some species between 4.0 – 5.5 and a slightly more alkaline (5.7) optimum for other species, but with a thin mantle and delayed root colonization at pH 8.0 (Wilcox and Wang 1987).

It has been reported that mycorrhizal roots may experience a shift in their pH range tolerance and exhibit better control over the pH of the surrounding rhizosphere, with both mycorrhizal and non-mycorrhizal roots releasing H^+ into the rhizosphere at a pH above 5.5, but in mycorrhizal roots, fewer OH^- were released per molecule of NO_3^- taken up to balance the ionic charge (Rygiewicz et al. 1984b). Additionally, mycorrhizal roots have exhibited a lower rate of K^+ efflux, and an upward shift in the pH at which Ca^{2+} efflux ceased (Rygiewicz et al. 1984b). Mycorrhizal roots may also be able to modify their environmental pH in response to the form of supplied nitrogen (Bago and Azcon-Aguilar 1997).

In pure culture, ectomycorrhizal fungi also have pH optima with respect to growth and performance. Although not much is known about their growth optima as free-living organisms, many species are considered to be pH-sensitive (Hung and Trappe 1983, Ohta 1990). *H. crustuliniforme* exhibited a pH optimum of 5 in buffered growth media (Littke et al. 1984), but a pH optimum of 8 in unbuffered media, with alkaline pH > 8 resulting in fungal growth inhibition (Hung and Trappe 1983). E-strain fungi have been reported to have a wide pH tolerance between 3.9 – 8.0 in Hagar agar medium, but in association with *Pinus sylvestris* in peat moss, their pH range is even wider, shifting to 2.2-12.6 (Mikola 1965).

H. crustuliniforme and *W. mikolae* are considered to be alkaliphilic and are tolerant to more alkaline conditions than other ECM species, with growth that increased at each pH tested between 4.1 – 6.8; although both species exhibited relatively slow growth, *W. mikolae* grew faster than *H. crustuliniforme* at all pH values tested, with optimal growth at 6.8 for both species (Kernaghan et al. 2002).

Ectomycorrhizal plants have shown reductions in nitrogen and nitrate uptake (Rygiewicz et al. 1984b, Andersson et al. 1997) and in glutamate and glutamine uptake (Chalot et al. 1995), with concurrent reductions in protease activity (Bending and Read 1995) within a pH range between 4.5 – 5.9, compared to nitrogen uptake and enzyme activity at lower pH. Part of the reduction in nitrogen uptake may also be due to nutrient immobilization by chemical reactions or by bacteria (Andersson et al. 1997). Bacterial communities in the soil are also pH-dependent as well as fungal species-dependent (Andersson et al. 1997). Another study showed that fungal enzymology changes with liming, stimulating certain enzymes in fungal tissue due to increased nitrogen solubility within a certain pH range (Dähne et al. 1995). In some AM species, mycorrhizal plants increased nitrogen uptake of roots in both acid and alkaline conditions compared to non-mycorrhizal plants, although the difference was greater at higher pH (Clark & Zeto 1996, Clark et al. 1999). Increased pH tolerance of mycorrhizal plants may be related to the ability of mycorrhizae to absorb less available minerals such as phosphorus under acidic conditions (Bagayoko et al. 2000, Ortas et al. 2004).

1.5. Biology and ecology of poplars

Much research has been undertaken with respect to the biology and ecology of *Populus* and its uses as a resource, from conservation strategies, interactions with pathogens, and cultivation of hybrids (Thomas and Robertson 1998, Stettler et al. 1996), to the multiple uses of *Populus* species for products made from wood fiber of both native and specially cultivated hybrid trees. Such uses include fuels, chemicals (Klass 1998), and use of trees in oilsands land reclamation (Khasa et al. 2002).

Little research with respect to mycorrhizal associations has been conducted with *Populus* tree species, compared to coniferous tree species (Khasa et al. 2002), although studies have shown that *Populus* species do not necessarily demonstrate much specificity for mycorrhizal species (Godbout and Fortin 1985). *Populus* clones are capable or forming both ECM and VAM associations (Khasa et al. 2002). Estimation of colonization rates is more difficult with *Populus* roots because of their lack of easily-identifiable features, such as the dichotomously-branching roots of conifers (Khasa et al. 2002).

1.5.1. *Populus tremuloides*

P. tremuloides Michx. is a fast-growing woody angiosperm with the widest geographical distribution of any native tree species in North America (Little 1979). It grows on a wide range of soils where annual precipitation exceeds evapotranspiration, although its growth is restricted in flooded soils (Perala 1990). It is found predominantly in Eastern- (Type 16) and Western-Aspen (Type 217) forest, and in White Spruce-Aspen (Type 251) forest, as classified by the Society of American Foresters (Eyre 1980). *P. tremuloides* grows best on well-drained, nutrient-rich soils with high quantities of organic matter, nitrogen, calcium, magnesium, and potassium; it is an important species in nutrient cycling because of its rapid growth and high nutrient requirements (Alban 1978, 1982). Soils under aspen-dominated stands have been found to have slightly higher pH and more available phosphorous, as well as sufficient nitrate for aspen growth, compared with adjacent soils under shrubs or herbaceous vegetation (Tew 1968). Aspen foliage is typically high in nutrients relative to associated coniferous tree species due to a high rate of nutrient uptake, and is therefore important in nitrogen cycling as aspen leaves readily decompose (Wengert et al. 1985). Aspen is a commercially valuable species, used in varying quantities for wood and paper products as pulp for paper, particleboard and flakeboard, structural lumber, studs, veneer, plywood, and shingles (Perala 1990).

1.5.2. *Populus balsamifera*

P. balsamifera L. is a fast-growing woody angiosperm that has the most northern distribution of native North American hardwood tree species (Zasada and Phipps 1990) with optimal growth on floodplains and on floodplain-associated sandy and silty soil types with fewer nutrients (Zasada and Phipps 1990). *P. balsamifera* is found in several forest types including balsam poplar, white spruce-aspen, white spruce, jack pine, aspen, red spruce-balsam fir, and northern white cedar (Eyre 1980). Commercially, *P. balsamifera* can be used for pulp, veneer, boxes, brackets, and structural lumber, but it is not as important as hybrid poplar, which is being used in commercial plantations for pulpwood, and planted for shelterwood, urban forestry, and soil stabilization projects (Zasada and Phipps 1990).

1.6. Study objectives

We know that mycorrhizal associations can be beneficial for plants, and we also know of many of the environmental stresses and conditions under which mycorrhizal fungi can benefit plant species. However, the literature indicates that we have little information in the following areas:

(1) The physiological and cellular mechanisms by which mycorrhizal fungi assist their plant hosts in mitigating the effects of environmental stresses, particularly in *Populus* tree species.

(2) The environmental and other conditions under which mycorrhizal fungi benefit their host plant species, as indicated by the experimental evidence that mycorrhizal fungi do not always form mutualistic associations with plants.

(3) The apparent contradiction that trembling aspen, an ammonium-sensitive tree species, can thrive in some mature forest types where ammonium is the predominant form of nitrogen in forest soils.

(4) The ecology and physiology of ectendomycorrhizal fungal species and ectendomycorrhizal associations, particularly with respect to their possible association with deciduous broadleaf tree species.

The main objectives of this study were to:

(1) Assess the long-term effects of different forms and levels of inorganic nitrogen (ammonium and nitrate) treatments on the growth, water relations, nitrogen assimilation, and nitrogen metabolizing activity of trembling aspen with and without mycorrhizal fungi.

(2) Assess the long-term effects of different forms of nitrogen on the growth and nitrogen metabolizing activity of a pure-culture ectomycorrhizal fungus grown on media at a range of different pH conditions.

(3) Examine the effects of the ectomycorrhizal fungus *Hebeloma crustuliniforme* on the water relations of trembling aspen exposed to a range of different pH conditions in the short-term.

(4) Examine the effects of an ectomycorrhizal fungus (*Hebeloma crustuliniforme*) and an E-strain fungus (*Wilcoxina mikolae* var. *mikolae*) on general water relations and root anatomy in balsam poplar.

I tested the hypotheses that:

(1) Trembling aspen will exhibit reduced growth and physiological function in the presence of ammonium due to its ammonium sensitivity.

(2) The ectomycorrhizal fungus *Hebeloma crustuliniforme* will increase the growth and enhance root water relations of trembling aspen subjected to unfavourable nitrogen treatments, where high levels of ammonium are present and where levels of total nitrogen are low.

(3) *Hebeloma crustuliniforme* will increase nitrogen nutrition and ammonium tolerance of trembling aspen by increasing nitrogen uptake and ammonium assimilation, and decreasing mortality, in mycorrhizal aspen seedlings.

(4) *Hebeloma crustuliniforme* will increase root water flux of trembling aspen under alkaline pH conditions by increasing aspen's tolerance for alkaline pH.

(5) The ectomycorrhizal fungus *Hebeloma crustuliniforme*, when grown in pure culture in the absence of a host plant, will demonstrate its ability to metabolize ammonium and to survive in alkaline pH conditions.

(6) Mycorrhizal fungi will increase root water relations in balsam poplar and trembling aspen by increasing root water flux.

(7) The E-strain fungus *Wilcoxina mikolae* var. *mikolae* will form an ectendomycorrhizal association with balsam poplar.

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CHAPTER II

Effects of ammonium and nitrate on growth and water relations of trembling aspen (*Populus tremuloides*) seedlings.

2.1. INTRODUCTION

Ammonium is the predominant form of inorganic nitrogen present in several types of mature, undisturbed boreal forest soils (Prieto-Fernandez et al. 1993, Nordin et al. 2001). The type of inorganic nitrogen may influence and be influenced by the plant community, with the ammonium-to-nitrate ratio being higher in coniferous (33:1) compared with deciduous (5:1) forests (Jerabkova et al. 2006). Because the form and level of nitrogen can be limiting factors for plant growth (Vitousek and Howarth 1991), many boreal plant species have adapted to their environment, with mature successional tree species, particularly conifers, exhibiting preferential uptake and assimilation of ammonium (Kronzucker et al. 1997, 2003). Some trees that prefer ammonium have demonstrated superior growth and nitrogen assimilation when ammonium, rather than nitrate, was exclusively supplied in greenhouse studies (Lavoie et al. 1992). Other trees have shown equivalent growth in either nitrate or ammonium (Flaig and Mohr 1992) unless ammonium concentrations became excessive (Olsthoorn et al. 1991, Griffin et al. 1995). However, in mature boreal forests, early-successional tree species, such as trembling aspen and Douglas-fir, preferentially assimilate nitrate (Min et al. 1999), which becomes more available and may become the dominant form of inorganic nitrogen in early-successional and disturbed boreal forest soils (Prescott 1997, Min et al. 1998). These species have been previously considered to be "ammonium-sensitive" because they have shown symptoms of excess ammonium accumulation and reduced growth in response to concentrations of supplied ammonium similar to ammonium concentrations that are present in native soils (Min et al. 1999, Kronzucker et al. 2003). In comparison, ammonium-tolerant species may also exhibit ammonium toxicity symptoms with exposure to much higher ammonium concentrations. It has been suggested that a physiological mechanism known as futile transmembrane ammonium cycling, resulting in uncontrolled ammonium uptake and loss and in ammonium accumulation in tissues, is the reason for the ammonium sensitivity in these species (Britto et al. 2001). However, there has been no research to date that provides an explanation for the ability of trembling aspen to survive and grow to maturity in late-successional boreal forests, where measured soil ammonium concentrations can be within the range of toxicity for some ammonium-sensitive species (Roelofs et al. 1988, Jerabkova et al. 2006). There are ecological implications for long-term growth of species which may be ammonium-sensitive, since agricultural and industrial emissions can have a significant impact on soil ammonium concentrations (Bijlsma et al. 2000, Valiela et al. 2000), which may eventually lead to localized species-specific declines in plant populations (de Graaf et al. 1998).

While a number of studies have examined and compared the long-term effects of nitrate and ammonium on growth and physiology in coniferous trees (for examples, see Arnold 1992, Lavoie et al. 1992, de Visser and Keltjens 1993, Kronzucker et al. 1997, Rothstein and Cregg 2005), only a small number of studies have been conducted regarding the long-term effects of nitrogen nutrition on growth and the physiology of *Populus* spp. (Marler et al. 2001, Choi et al. 2005, van den Dreissche et al. 2005, DesRochers et al. 2006). However, these studies made no differentiation between nitrate and ammonium fertilization. It has been established that trembling aspen prefers nitrate to ammonium in the short term (< 28 days) at the cellular level, and that ammonium may have a negative effect on some physiological functions in aspen (Min et al. 1998, 1999), but not much is known about the longer-term (> 1 month) effects of nitrate and ammonium concentrations on trembling aspen seedlings. It is also not known what range of ammonium concentrations might be considered toxic for trembling aspen or whether these plants can recover from initially-supplied high ammonium concentrations.

The objectives of this study were: 1) to measure and compare the anatomical and physiological effects of different levels of nitrate and ammonium supplied to trembling aspen seedlings over an initial one-month period, and; 2) to observe any potential recovery of trembling aspen seedlings with a reduction in supplied ammonium concentrations over a subsequent one-month period. It was predicted that nitrate treatments would result in greater growth, stomatal conductance, and root hydraulic conductivity than the ammonium treatments. Recovery of trembling aspen following a reduction in the supplied ammonium concentrations was expected to occur, provided that the concentrations of ammonium supplied in the different treatments in this study were not sufficient to result in mortality.

2.2. MATERIALS AND METHODS

2.2.1. Plant culture

One-year old greenhouse-grown *Populus tremuloides* seedlings (seedlot BOR 5-65-3-4-03) from Bonnyville Forest Nursery (Bonnyville, AB, Canada) were used for the study. Seedlings were grown in commercial soil mix in 415D styroblocks™, fertilized, then hardened, and removed from styroblocks with intact soil plugs. Soil plugs were wrapped in plastic, and seedlings were stored at 4°C in the dark prior to experimental use. All seedlings were approximately 12-20 cm tall, and 3-6 mm in stem diameter at the root collar.

Dormant seedlings were brought out of cold storage, dead roots were trimmed from root systems, roots were washed free of the potting media, and the seedlings were placed in 30 L solution culture tubs for ten days with aerated deionized water in a controlled environment growth chamber. After one week, most seedlings had flushed, with small leaves visible. All seedlings that did not show signs of flushing after 10 days were removed from the experiment. During flushing and for the duration of the experiment, growth chamber conditions were 60% RH, 18 hr photoperiod, 22°C/18°C day/night, 300 μmol m^{-2} s^{-1} PAR with full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

2.2.2. Solution culture set-up

The solution culture set-up consisted of several 30 L opaque plastic tubs with lids. Into each lid, 18 x 3.8 cm holes were cut, so that seedling roots could be slipped into the nutrient solution through the lid. There were a maximum of 18 seedlings per tub. Snug-fitting foam plugs were fitted around the stems, and inserted into the holes to hold the stems in place while the roots were immersed in solution, with the stems protruding through the lid. All tubs had spouts installed into their sides to facilitate drainage, aeration, and circulation of nutrient solution via plastic tubing.

There was one tub for each nutrient treatment, connected with a circulating pump (Models 3E-12R, 3.2 A, and Type U21, 1.4 A, Little Giant Pump Co., Oklahoma City, OK, USA) that circulated nutrient solution through tubs with Nalgene 180 non-toxic PVC Vi grade autoclavable/lab/FDA/USP tubing (Fisher Scientific). An aeration pump (model SPP-40 GJ-L with 12-valve attachment, Rolf C. Hagen Inc., Montreal, PQ, Cda) was connected to all the tubs via Fisherbrand 3/16" ID x 1/16" wall flexible tubing (Fisher Scientific) to maintain continuous aeration of the solutions (mean dissolved O_2 of 5.52 mg L^{-1}, SE=0.371, n=14). Dissolved O_2 values > 5 mg L^{-1} while root systems were immersed in treatment solutions were considered to provide adequate aeration. The solution culture tubs, tubing, and pumps were cleaned once per week to prevent algal and bacterial buildup.

2.2.3. Treatments

After ten days of flushing, aerated water was replaced with one of seven nutrient solution treatments. These were no nitrogen (N-0), low nitrate (N-L), medium nitrate (N-M), high nitrate (N-H), low ammonium (A-L), medium ammonium (A-M), and high ammonium (A-H). In the N-L, N-M, and N-H treatments, nitrate was the only source of supplied nitrogen. In the A-L, A-M, and A-H treatments, ammonium was the only source of supplied nitrogen. The concentrations of nitrogen represented by these levels were chosen because N-0 represents a negative nitrogen control. The low, medium, and high concentrations during the 4-week period represent average, higher than average, and excessively higher than average nitrogen concentrations found in typical boreal forest soils, respectively.

Seedlings were exposed to these nutrient concentrations for 4 weeks. After 4 weeks, seedlings from all the treatments were harvested for measurements. For the N-0, A-L, A-M, and A-H treatments, remaining seedlings continued to be provided with fresh solutions on a weekly basis for the remainder of the experiment. For the ammonium-containing treatments, ammonium concentrations were reduced by half for the remaining

5-8 weeks of the experiment, to gauge seedling response and recovery from the higher ammonium concentrations to which they were exposed during the first four weeks. After 8 weeks, the remaining seedlings were harvested for measurements.

The nutrient composition of the solutions was based on modified Hoagland's solution (Hoagland and Arnon 1950) with individual nutrient concentration equal to or greater than 100% Hoagland's solution to ensure sufficient nutrient concentrations and prevent deficiency without producing severe toxicity. Concentrations of nitrogen in applied 4-week treatments were: N-0 (0 mM); N-L (4 mM); N-M (8 mM); N-H (16 mM); A-L (4 mM); A-M (8 mM); A-H (16 mM). Concentrations of nitrogen in applied 8-week treatments were: N-0 (0 mM); A-L (2 mM); A-M (4 mM); A-H (8 mM). Nitrate was provided as $Ca(NO_3)_2$. Ammonium was provided as $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$. The concentrations of nutrients other than nitrogen for both the 4-week and 8-week treatments were: N-0 (K=6 mM, PO_4=6 mM, SO_4=6 mM, Ca=4 mM, Mg=2mM); LA (K=6 mM, PO_4=5 mM, SO_4=8 mM, Ca=4 mM, Mg=2mM); MA (K=6 mM, PO_4=6 mM, SO_4=9 mM, Ca=4 mM, Mg=2mM); HA (K=6 mM, PO_4=7 mM, SO_4=11.5 mM, Ca=4 mM, Mg=2mM). Concentrations of micronutrients were the same as for 100% modified Hoagland's solution for both 4- and 8-week treatments, and were supplied in mg L^{-1} as follows: Fe-EDTA (5), B (0.5), Mn (0.5), Zn (0.05), Cu (0.02), Mo (0.01). The pH of nutrient solutions was adjusted every other day with either H_2SO_4 or KOH to 5.0-5.5.

Electrical conductivity was measured for all solutions (FisherBrand Conductivity Meter MDL C33), temp= 23.8 C, mean E_c=1.30µS/cm, SE=0.048, n=14. Samples of treatment solutions in which plants were growing were randomly collected from the growth tubs for all treatments during the experiment and frozen at -20°C until they could be analyzed. To verify that no bacterial ammonification or nitrification was occurring in treatment solutions that could potentially add excess ammonium or nitrate ions to nitrate- or ammonium-containing treatments, respectively, solution samples were randomly collected and analyzed for ammonium and nitrate ions using a Technicon Autoanalyzer (Technicon Industrial Systems 1973a,b).

2.2.4. Measurements

At the end of the 4- and 8-week treatments, plants were harvested in random order and measurements were taken. Percent mortality for all treatments was calculated at the end of the experiment. Most measurements described below were obtained for each seedling in both the 4-week and 8-week groups. Activation energy and upper and lower leaf chlorophyll analysis were collected on a separate group of seedlings from the 4-week (N-0, N-L, and A-L treatments) and 8-week (N-0, A-L treatments) groups.

2.2.4.1. Stomatal conductance and shoot water potential

Stomatal conductance (g_s) was measured *in situ* for intact plants within the growth chamber with an LI-1600 steady-state porometer (LI-COR, Lincoln, NE, USA), using a 2-cm^2 aperture setting with the broadleaf attachment. Leaves with minimal or no necrosis at approximately the mid-region of the stems which still contained living leaves were selected for measurements.

Shoot water potential (Ψ_w) measurements were conducted using a Scholander pressure chamber, as described by Siemens and Zwiazek (2003). Larger shoots (> 16 cm) that were too big to fit into the chamber were trimmed from the base before being placed into the pressure chamber.

2.2.4.2. Shoot height, stem diameter, and leaf area

Seedling height measurements were taken in intact plants, from the base of the stem to the shoot tip. Stem diameter measurements were taken with calipers at the base of the stem.

Projected leaf area measurements were taken for all of the leaves from each plant after removing the leaves and measuring their total surface area with a LI-3100C leaf area meter (LI-COR, Lincoln, NE, USA). For plants that required upper and lower leaf fresh weight measurements, leaves from the top half of the stem, as determined by stem height, were removed and weighed separately from leaves from the lower half of the stem. Shoot fresh weights were determined for all leaves plus the entire shoot from the shoot apex to the base of the stem. Shoots and leaves were then placed in paper bags and dried for 72 hrs at 65°C in a drying oven (Despatch PBC 224, Despatch Industries, Inc., Minneapolis, MN, USA) and weighed.

2.2.4.3. Chlorophyll and total nitrogen analysis of leaves

Leaves from the upper half of the stem were randomly selected for chlorophyll analysis. Five leaf discs (total area of five discs = 1.925 cm^2) were cut from each plant and weighed. The discs were placed in light-proof tubes with 16 mL 80% acetone for 24 h and extracts analyzed with a Beckman spectrophotometer as outlined in Azia and Stewart (2001).

For a subsection of plants from the 4-week N-0, A-L, and N-L treatments, and the 8-week N-0 and A-L treatments, stems of each plant were divided by length into an upper and a lower section. Upper and lower leaves were harvested and analyzed separately for chlorophyll measurements.

Total leaf % N was determined for all plants after collecting and rinsing subsamples of leaves from each plant with deionized water, and immediately freezing leaf samples in liquid nitrogen for storage at -80 °C. Samples were then removed from storage, placed in paper bags and dried at 60 °C for 72 h in the drying oven. Dried leaf samples were weighed to ensure a minimum of 1 g per sample, ground with a Wiley mill until samples could be passed through a 20 mesh sieve. Samples were analyzed for total nitrogen by Dumas flash combustion reaction, using a Carlo-Erba NA 1500 analyzer (Carlo-Erba Instruments, Milan, Italy).

2.2.4.4. Root hydraulic conductivity and PTS_3 exudate concentrations

To measure root hydraulic conductivity, root systems were excised with approximately 2 cm of stem remaining, and placed in Scholander pressure chambers (PMS Instruments, Corvallis, OR) in an aqueous solution of 0.02% trisodium 3-hydroxy-5,8,10-pyrenetrisulfonic acid (PTS_3). PTS_3 is a fluorescent tracer dye restricted to the apoplastic pathway of water movement (as discussed by Steudle and Peterson 1998) which has been used to measure relative changes in water transport through the apoplast (Siemens and Zwiazek 2003, Schaider et al. 2006). The solution was continuously aerated with a magnetic stir bar using a magnetic stirrer placed underneath the pressure chamber. Root water flow rate (Q_v, $m^3\ s^{-1}$) was measured for a minimum of 20 minutes at hydrostatic pressures of 0.3, 0.6, and 0.9 MPa, a range within which changes in Q_v are known to be linear with pressure changes for aspen seedlings (Siemens and Zwiazek 2003, 2004). A minimum of 10-minute intervals between Q_v measurements at each increasing pressure were maintained to stabilize Q_v values. Once initial root water flux (J_v, $m^3\ s^{-1}\ MPa^{-1}$) measurements were made, root systems were de-pressurized and $HgCl_2$ solution was added to the PTS_3 solution to reach a final concentration of 0.05 mM. Root systems were re-pressurized for 30 minutes at 0.3 MPa, following which a second set of J_v values was collected at the same three hydrostatic pressures.

Root volumes were measured for each root system using volume displacement of water in a graduated cylinder, as described in Voicu and Zwiazek (2004). Root volumes were used to calculate L_{pr} ($s^{-1}\ MPa^{-1}$) from J_v before and after the addition of $HgCl_2$.

Xylem exudate samples were collected from pressurized roots following both sets of J_v measurements for each root for measurement of xylem PTS_3 concentration. Samples were diluted with water and measured for PTS_3 concentration against a PTS_3 standard curve using a Sequoia-Turner 450 spectrofluorometer (Apple Scientific, Chesterland, OH, USA) with a 405 nm excitation and 515 nm emission spectrum (Skinner and Radin 1994).

2.2.4.5. Root and shoot fresh weights

Root and shoot fresh weights were determined after trimming the remaining stem segment from roots, and washing and removing excess water with paper towels.

2.2.4.6. Activation energy

For a subset of plants from the 4-week N-0, N-L, and A-L treatments, and 8-week N-0 and A-L treatments, activation energy (E_a, kcal mol^{-1}) was measured in excised root systems using a Scholander pressure chamber (Wan and Zwiazek 1999). The temperature of the aqueous root bathing solution was controlled with a circulating water bath (Haake C, F3 digital control, Thermo Electron Corp., W. Germany) connected to hollow copper coils that were fitted through the pressure chamber lid and inserted into the root bathing solution. A digital thermometer probe was inserted into the pressure chamber through the lid to monitor temperature changes of the root bathing solution. Q_v measurements were collected at a constant 0.3 MPa hydrostatic pressure for a minimum of 20 minutes at each decreasing temperature (25, 20, 15, 10, and 5°C), with a minimum of 15 minutes interval between each Q_v measurement. Activation energy was calculated from Arrhenius plots of the slope of $\ln(L_{pr})$ and temperature $(^{\circ}K)^{-1}$ using the following equation:

$$E_a = -\,[R \times (\ln K_2 - \ln K_1)/(T_2^{-1} - T_1^{-1})]$$

where $R = 1.987 \times 10^{-3}$ kcal mol^{-1} T^{-1}

$K_2 = L_{pr}$ at point 2

$K_1 = L_{pr}$ at point 1

T_2 = temperature (°K) at point 2

T_1 = temperature (°K) at point 1

The designations "point 2" and "point 1" in the equation refer to the end-point locations on the Arrhenius plots between which there was a linear relationship.

2.2.4.7. Statistical analysis

All data were analyzed with SAS 9.1. (SAS Institute, North Carolina, USA) to determine statistically significant ($p \leq 0.05$) differences between treatment combinations of nitrogen forms and levels as well as between treatment durations for the ammonium treatments. All data were first tested for normality of distribution and homogeneity of variance. Residuals from the data sets were calculated and used to graph normal probability plots and box plots, to determine if there were any statistical outlier values in the data sets. Outliers, if any, were removed from the data sets.

2.2.4.7.1. Four-week measurements

Response variables (described in sections 2.2.4.1. – 2.2.4.5.) from nitrate and ammonium treatments were analyzed for statistically-significant differences. To compare the effect of nitrogen to the effect of no nitrogen, all nitrogen-containing treatments were considered as separate treatments and statistically compared to the N-0 treatment (no nitrogen) using Dunnett's test. To statistically compare the effects of different N-containing treatments to each other (excluding N-0), a MIXED ANOVA model of a 2 (nitrogen form) x 3 (nitrogen level) factorial experiment within a randomized complete block (RCB) design was used. A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment group harvested per day. Therefore, the days of measurements were the blocks.

The statistical model used for the RCB two-factor design was:

$Y_{ijkl} = u + N_i + L_j + N_iL_j + B_k + e_{ijkl}$, where:

Y_{ijkl} = value of individual observation (*i*=nitrogen, *j*=level, *k*=block, *l*=observation)

u = overall mean of observations

N_i = effect of i^{th} treatment (*i* = form of nitrogen)

L_j = effect of j^{th} treatment (*j* = level of nitrogen)

N_iL_j = interaction effect of i^{th} and j^{th} treatments

B_k = effect of k^{th} block (*k*=day of measurement)

e_{ijkl} = random error

4-week E_a measurements (described in section 2.2.4.6) and 4-week upper and lower leaf chlorophyll analysis measurements (described in section 2.2.4.3) were analyzed using a MIXED single-factor (nitrogen) ANOVA model RCB design.

The statistical model used for the single-factor (nitrogen) RCB design was:

$Y_{ijk} = u + N_i + B_j + e_{ijk}$, where:

Y_{ijk} = value of individual observation (*i*=nitrogen, *j*= block, *k*=observation)

u = overall mean of observations

N_i = effect of i^{th} treatment (*i* = form of nitrogen)

B_j = effect of j^{th} block (*j*=day of measurement)

e_{ijk} = random error

Comparisons from the Dunnett's test ($\alpha=0.05$) and MIXED ANOVA tests were conducted using least-squares means. For MIXED ANOVA, corresponding Tukey-adjusted p-values were used to further examine differences among the six treatment combinations (N form x level) using pre-planned comparisons ($\alpha=0.05$). The slice function was used to analyze and interpret interactions between main effects that were statistically significant ($\alpha=0.05$) for multi-factor MIXED ANOVA. The results of all statistical analyses are presented in figures and tables at the end of this chapter.

2.2.4.7.2. Eight-week measurements

Response variables from ammonium treatment measurements (sections 2.2.4.1. – 2.2.4.5.) were analyzed for statistically-significant differences. To statistically compare the effects of different levels of ammonium treatments to each other (including N-0), a MIXED single-factor (level of nitrogen) ANOVA RCB model was used. A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment group harvested per day. Therefore, the days of measurements were the blocks.

The statistical model used for the RCB single-factor design was:

$Y_{ijk} = u + N_i + B_j + e_{ijk}$, where:

Y_{ijk} = value of individual observation (*i*=ammonium, *j*= block, *k*=observation)

u = overall mean of observations

N_i = effect of i^{th} treatment (*i* = level of ammonium)

B_j = effect of j^{th} block (*j*=day of measurement)

e_{ijk} = random error

The 4-week and 8-week response variables from the ammonium treatment measurements (sections 2.2.4.1. – 2.2.4.5.) were analyzed for statistically-significant differences. To compare the effects of the six different ammonium level and week combinations, a MIXED ANOVA model 2 (week) x 3 (ammonium level) factorial RCB design was used (excluding N-0). A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment group harvested per day. Therefore, the days of measurements were the blocks.

The statistical model used for the RCB two-factor design was:

$Y_{ijkl} = u + N_i + W_j + N_iW_j + B_k + e_{ijkl}$, where:

Y_{ijkl} = value of individual observation (*i*=ammonium, *j*=week, *k*=block, *l*=observation)

u = overall mean of observations

N_i = effect of i^{th} treatment (*i* = ammonium treatment)

W_j = effect of j^{th} treatment (*j* = week)

N_iW_j = interaction effect between i^{th} and j^{th} treatments

B_k = effect of k^{th} block (*k*=day of measurement)

e_{ijkl} = random error

Eight-week E_a measurements (described in section 2.2.4.6) and 8-week upper and lower leaf chlorophyll analysis measurements (described in section 2.2.4.3) were analyzed using a MIXED single-factor (nitrogen) ANOVA model RCB design. A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment group harvested per day. Therefore, the days of measurements were the blocks.

The statistical model used for the single-factor (level) RCB design was:

$Y_{ijk} = u + N_i + B_j + e_{ijk}$, where:

Y_{ijk} = value of individual observation (*i*=nitrogen, *j*= block, *k*=observation)

u = overall mean of observations

N_i = effect of i^{th} treatment (*i* = level of ammonium)

B_j = effect of j^{th} block (*j*=day of measurement)

e_{ijk} = random error

The 4- and 8-week N-0 and A-L treatment measurements for E_a (section 2.2.4.6) and upper and lower leaf chlorophyll (section 2.2.4.3.) were compared against each other by week using a 2 (ammonium) x 2 (week) factorial mixed ANOVA model RCB design. A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment group harvested per day. Therefore, the days of measurements were the blocks.

The statistical model used for the 2-factor RCB design (4- and 8-week comparisons) was:

$Y_{ijkl} = u + N_i + W_j + N_iW_j + B_k + e_{ijkl}$, where:

Y_{ijkl} = value of individual observation (i=ammonium, j=week, k=block, l=observation)

u = overall mean of observations

N_i = effect of i^{th} treatment (i = level of ammonium)

W_j = effect of j^{th} treatment (j = week)

N_iW_j = interaction effect between i^{th} and j^{th} treatments

B_k = effect of k^{th} block (k=day of measurement)

e_{ijkl} = random error

Comparisons from the MIXED ANOVA tests were conducted using least-squares means. For MIXED ANOVA, corresponding Tukey-adjusted p-values were used to further examine differences among the different treatment combinations using pre-planned comparisons (α=0.05). The slice function was used to analyze and interpret interactions between main effects that were statistically significant (α=0.05) for multi-factor MIXED ANOVA. The results of all statistical analyses are presented in figures and tables at the end of this chapter.

2.3. RESULTS

2.3.1. Stomatal conductance and shoot water potential

Four-week (N-0 control and all nitrogen treatments) and 8-week (N-0 control and all ammonium treatments) least-squares means for stomatal conductance (g_s) are shown (Fig. 2.1A). Four-week g_s means differed significantly with different concentrations of both nitrate and ammonium (Tab. 2.1). At 4 weeks, the N-H treatment had a higher g_s in comparison with the other nitrate and ammonium treatments and N-0 (55.2 ± 14.9 mmol m^{-2} s^{-1}) (Fig. 2.1A). The A-M treatment had the highest g_s of the 4-week ammonium treatments, but was not significantly different from N-0. Except for N-H and A-M, all other treatments had values lower than N-0. At 8 weeks, A-L treatment showed a significant two-fold increase in g_s relative to its 4-week treatment (Fig. 2.1A). The 8-week A-M and A-H g_s means were significantly lower than those of A-L, but they did not significantly differ from the 4-week treatment. The g_s means for the 8-week A-L and A-M treatments were significantly higher than the 8-week N-0 treatment. The A-H treatment had the lowest g_s means at both 4 weeks and 8 weeks.

At 4 weeks, shoot water potential (Ψ_w) for all treatments did not differ significantly from that of N-0 for most of the nitrogen treatments, with the exception of N-M and A-H which were at least two-fold significantly lower than N-0. Only the means between the 4-week ammonium treatments were found to be significantly different (Tab. 2.1). Eight-week Ψ_w means from the ammonium treatments did not differ significantly from N-0, although the mean of A-H was lower than that of A-M. There were no significant differences between 4- and 8-week means for any ammonium treatment. Least-squares means and standard errors for 4- and 8-week shoot Ψ_w are shown (Tab. 2.2).

2.3.2. Leaf area, root:shoot ratio, and stem diameter

Total leaf area least-squares means and standard errors are shown (Fig. 2.1B). Four-week surface area means differed significantly with concentrations of both nitrate and ammonium (Tab. 2.1). At 4 weeks, N-H had the significantly highest leaf area relative to all other nitrogen treatments and N-0 (103.0 ± 17.0 cm^2) (Fig. 2.1B). Although N-L, N-M, A-L, and A-M treatments had significantly higher leaf areas than N-0, the differences were not significant between treatments. At 8 weeks, leaf areas for A-L, A-M, and A-H treatments decreased slightly relative to the 4-week treatment, but the decrease was not significantly different. They were also not significantly different from the 8-week N-0 treatment (98.6 ± 4.6 cm^2). A-H treatment had the lowest leaf area at both 4 weeks and 8 weeks.

Least-squares means and standard errors for root:shoot ratios are shown (Fig. 2.1C). Four-week ratios differed significantly between concentrations of both nitrate and ammonium (Tab. 2.1). At 4 weeks, N-L, A-M, and N-H had the lowest fresh weight root:shoot ratios, which were significantly lower than the other nitrogen treatments and N-0 (1.7 ± 0.4) (Fig. 2.1C). A-H had the highest ratio. At 8 weeks, the ratio for A-M was significantly higher than the 4-week mean ratio, but the 8-week ratios for A-L and A-H remained relatively unchanged. 8-week ratios were not significantly different from N-0 (2.3 ± 0.3), which had the highest root:shoot ratio.

Seedling stem diameters showed a similar trend as for g_s and leaf area. At 4 weeks, mean stem diameters differed significantly between concentrations of both nitrate and ammonium (Tab. 2.1), showing increases with increasing nitrate concentrations, and decreases with increasing ammonium concentrations relative to N-0. At 8-weeks, mean stem diameters for all ammonium treatments showed a slight increase relative to their 4-week means, but the increase was only significant for A-M. Least-squares means and standard errors for stem diameter are shown in Tab. 2.2.

2.3.3. Root hydraulic conductivity

Least-squares means for root hydraulic conductivity (L_{pr}) are shown for all 4-week treatments and 8-week ammonium treatments (Fig. 2.2A). Four-week means differed significantly with different concentrations of both nitrate and ammonium (Tab. 2.1). Initial L_{pr} prior to the addition of $HgCl_2$ increased significantly by 50% in 4-week N-H and A-M treatments, and decreased significantly by 10-fold in the A-H treatment relative to N-0 ($8.92 \times 10^{-5} \pm 1.71 \times 10^{-5}$ s^{-1} MPa^{-1}) (Fig. 2.2A). L_{pr} values for N-L, N-M, and A-L decreased slightly relative to N-0. At 8 weeks, L_{pr} for A-L and A-M were 4-fold higher than N-0 ($2.64 \times 10^{-5} \pm 3.00 \times 10^{-6}$ s^{-1} MPa^{-1}), but not significantly different from each other or compared with the 4-week treatment (Fig. 2.2A). However, A-H showed a significant increase compared to the 4 week treatment, even though it was not significantly different from the 8-week N-0 mean.

Following the addition of $HgCl_2$ to roots, there was a general decrease in L_{pr} for most treatments, which is shown as normalized L'_{pr} for both 4 weeks and 8 weeks (Fig.

2.2B). For the 4-week means, there were only significant differences between different concentrations of the nitrate concentrations (Tab. 2.1). Normalized L_{pr} is represented as a percentage of corresponding L_{pr} values prior to the addition of $HgCl_2$ to roots, indicated by the line at the 100% mark, therefore decreases from untreated L_{pr} values are represented by values less than 100%. Only the 4-week N-M treatment had a significantly higher normalized L_{pr} than that of N-0 (64.2 ± 14.1%), which was also the only treatment with a normalized L_{pr} >100%. N-H had the lowest normalized L_{pr} of all the 4-week treatments, which was significantly lower than that of N-0 and N-M. Four-week N-L, A-L, A-M, and A-H all showed slight increases in normalized L_{pr} compared with N-0, but were not significantly different. Eight-week normalized L_{pr} increased in A-L and decreased in A-M and A-H treatments, but this did not represent a significant difference from the 4-week means, and they were not significantly different from each other or from N-0 (89.3 ± 8.3%).

2.3.4. Xylem PTS_3 concentration

Least-squares means for PTS_3 concentrations in xylem exudate are shown for 4- and 8-week treatments (Fig. 2.3A). The initial PTS_3 concentrations prior to the addition of $HgCl_2$ were significantly different between the different nitrate treatments (Tab. 2.1). Mean PTS_3 concentrations increased with increasing nitrate concentration to a maximum of $2.80 \times 10^{-3} \pm 7.09 \times 10^{-4}$ % for the N-H treatment, which was almost 3-fold higher than N-0 ($1.03 \times 10^{-3} \pm 1.54 \times 10^{-4}$ %) (Fig. 2.3A). The concentrations also significantly increased with decreasing ammonium concentration relative to N-0, with A-L having the highest mean concentration of all the ammonium treatments ($1.87 \times 10^{-3} \pm 3.68 \times 10^{-3}$ %). Eight-week A-L and A-M concentrations increased 2-fold relative to the 4-week treatment duration. With A-L, the increase was significantly higher compared to the 4-week mean as well as the 8-week N-0 mean ($5.38 \times 10^{-4} \pm 6.04 \times 10^{-5}$ %). Compared to the A-L and A-M treatments, the mean PTS_3 concentration of A-H remained relatively unchanged between the 4-week and 8-week treatment durations.

Following the addition of $HgCl_2$ to roots, PTS_3 concentrations increased in all 4- and 8-week treatments relative to their initial PTS_3 concentrations, although the differences were only significant between the different nitrate concentrations (Tab. 2.1). This relative increase is expressed as normalized PTS_3 concentrations (Fig. 2.3B), relative to corresponding PTS_3 concentrations prior to the addition of $HgCl_2$ to roots (indicated by the line at 100%). For the 4-week nitrate and ammonium treatments, PTS_3 did not increase significantly relative to N-0 (191.1 ± 37.9 %), with the exception of N-L, which had a significant 2-fold increase in PTS_3 concentration. Eight-week PTS_3 concentrations decreased in all ammonium treatments relative to their 4-week means, although this decrease was only significant for A-L. However, none of the 8-week means were significantly different from each other or from N-0 (150.6 ± 24.9 %).

2.3.5. Seedling mortality

Seedling mortality values for the entire experiment are shown (Fig. 2.4A). At 4 weeks, mortality decreased slightly from N-0 (10.3%) with increasing nitrate concentration to a minimum mortality for N-M (3.3%) and N-H (3.5%), and increased substantially with increasing ammonium concentration to a maximum mortality for A-H (44.0%). At 8 weeks, mortality values increased slightly for A-M, decreased slightly for A-L, and decreased by almost half for A-H (25.0%), relative to their 4-week values. However, mortality for all 8-week ammonium treatments was still higher than for N-0 (0%) (Fig. 2.4A).

2.3.6. Total leaf nitrogen

Four- and 8-week least-squares means for total leaf nitrogen are shown (Fig. 2.4B). Four-week leaf nitrogen means for all nitrate and ammonium treatments were significantly higher than N-0 (1.6 ± 0.01%), with N-H and A-H having the highest values. There were significant differences between the different concentrations of nitrate and of ammonium (Tab. 2.1). Eight-week nitrogen concentrations decreased for all ammonium treatments compared to 4-week concentrations, but this decrease was only significant for A-H. Eight-week nitrogen concentrations were still significantly higher compared with 8-week N-0 (1.4 ± 0.02 %).

2.3.7. Leaf chlorophyll

At 4 weeks, total chlorophyll content of leaves showed the same trend as total leaf area and showed significant increases with increasing nitrate concentrations to a maximum of 3.23 ± 0.28 mg g^{-1} FW for N-H, relative to N-0 (1.55 ± 0.15 mg g^{-1} FW) at 4 weeks (Tab. 2.2). None of the ammonium treatments were different from N-0 (Tab. 2.1). At 8 weeks, there were no significant changes in total chlorophyll concentrations compared to 4-week means, although A-L and A-M were significantly higher than N-0 (0.87 ± 0.32 mg g^{-1} FW) (Tab. 2.2).

Least-squares means for 4- and 8-week chlorophyll b:a ratios are shown (Fig. 2.4C). Mean ratios did not change significantly with nitrate treatments relative to N-0 (0.24 ± 0.006) but did increase significantly for A-M and A-H treatments. The differences between ammonium, but not between nitrate treatments, were statistically significant (Tab. 2.1). Eight-week chlorophyll b:a ratios for all ammonium treatments were not significantly lower than their 4-week means,. Although A-H had the highest 8-week mean ratio, only the 8-week mean for A-L was significantly different from N-0 (0.290 ± 0.00478).

Least-squares means of chlorophyll b:a ratios for 4-week (N-0, N-L, L-A) and 8-week (N-0, A-L) treatments are shown (Fig. 2.5A, top leaves; Fig. 2.5B, bottom leaves). For the top leaves, only the 4-week A-L mean was significantly lower than N-0 (0.285 ± 0.0202) (Fig. 2.5A). At 8 weeks, there was no difference between the mean top leaf ratios of A-L and N-0 (0.145 ± 0.0731), although the 8-week means showed a non-significant decrease compared with the 4-week means (Fig. 2.5A).

In the lower leaves, the mean chlorophyll b:a ratios showed a similar trend as for the means ratios in the top leaves. Only the 4-week mean ratio for A-L was significantly lower than N-0 (0.226 ± 0.0102) (Fig. 2.4B). The 8-week mean ratios of the lower leaves decreased in both A-L and N-0 treatments relative to their 4-week means, but the differences were not significant. There was also no difference between the 8-week A-L and N-0 means (0.225 ± 0.0631) (Fig. 2.5B).

2.3.8. Activation energy

Least-squares means of activation energy (E_a) are shown for 4-week (N-0, N-L, A-L) and 8-week (N-0, A-L) treatments (Fig. 2.5C). Although mean E_a increased in N-L and decreased in A-L treatments compared to N-0 (5.49 ± 0.877 kcal mol^{-1}), the differences were not significant at 4 weeks. The 8-week mean E_a of A-L decreased relative to its 4-week mean, but the difference was not significant. The 8-week A-L mean was also not significantly lower than that of N-0 (4.27 ± 0.730 kcal mol^{-1}).

2.4. DISCUSSION

2.4.1. Four-week treatment

From the 4-week nitrogen treatments, it was apparent that aspen was capable of utilizing and assimilating high (16 mM) concentrations of nitrate (N-H treatment) when it was provided without any evidence of decreased growth or increased mortality. At these high concentrations, aspen preferred nitrate over ammonium. The N-H treatment resulted in the largest mean stem diameter, leaf area, and nitrogen assimilation in leaves, and the lowest percent mortality and root:shoot ratio. N-H also resulted in the highest g_s, L_{pr}, and Hg-responsive AQP-mediated root water flux indicated by the greatest reduction following the addition of $HgCl_2$, compared with all the other nitrate and ammonium treatments in this study. For many of the measurements taken in this study, the differences between the N-H treatment and the N-L and N-M treatments were significant. The large differences between the measured parameters of the N-H treatment compared with the N-M and N-L treatments were not surprising, since trembling aspen is thought to have ecological adaptations that have resulted in a preference for nitrate over ammonium as a source of inorganic nitrogen (Min et al. 2000, Kronzucker et al. 2003). Therefore aspen growth continued to increase with increasing nitrate concentrations.

Lower concentrations of nitrate (N-L, N-M treatments) resulted in modest increases in leaf area, nitrogen assimilation, and mortality, compared to N-0. However, for many of the measured parameters (including shoot Ψ_w, total chlorophyll, chlorophyll b:a ratio, g_s, stem diameter, root:shoot ratio, L_{pr}, and apoplastic flux indicated by PTS_3 concentration), there were few differences between the N-L and N-M treatments even though the nitrate concentration of the N-M treatment (8 mM) was twice that of the N-L treatment (4 mM). The small differences between the N-L and N-M treatments, and the large difference between these treatments and N-H, seem to suggest that the nitrate concentrations provided in the N-L and N-M treatments under these experimental conditions may have been insufficient for increased growth of aspen in this study. Carvajal et al. (1996) reported reduced g_s measurements in nitrogen-deficient plants, consistent with the N-L and N-M treatments in this study. Little is currently known about long-term nitrate metabolism and the effects of nitrate deficiency in aspen, since most studies of aspen nitrogen nutrition have been ecological in nature and have consisted of observations of nitrogen-fertilized aspen in managed stands (van den Dreissche et al. 2005, DesRochers et al. 2006). However, one such study reported that the N fertilization efficiency of aspen was low, with a low percentage of applied N incorporated into shoot biomass (Jug et al. 1999). In the current study, it is possible that the Piper-Steenbjerg effect (Wikström 1994) may have occurred at these lower concentrations. The presence of nitrate could have stimulated plant growth or physiological process, such as photosynthesis or protein production, which could have resulted in increased utilization of and demand for assimilated nitrate. This could have resulted in a state of nutrient deficiency created by the increased growth or physiological function that could not be sustained by supplied concentrations of nitrate (N-L, N-M treatments). This may also explain why leaf nitrogen concentrations in the N-L and N-M treatments were the same.

Within the 4-week ammonium treatments, it was apparent that aspen seedlings did not respond to increasing ammonium concentrations as they did to increasing nitrate concentrations. Higher concentrations of ammonium resulted in reduced growth, and increased symptoms of toxicity and mortality, indicated by decreases in g_s, leaf area, apoplastic flux, stem diameter, total chlorophyll, shoot Ψ_w, and root water flux through Hg-responsive AQPs. There were also increases in mortality, root:shoot ratio, L_{pr}, and chlorophyll b:a ratio with increasing ammonium concentrations. These results suggest that aspen were sensitive to high ammonium concentrations, compared to equivalent nitrate concentrations. These results were somewhat consistent with previous studies that demonstrated increased ammonium accumulation in aspen (Min et al. 1998, 1999, Britto and Kronzucker 2002). However, these previous studies did not observe toxicity symptoms in the long term, apart from increased ammonium accumulation, because long-term exposure and effects of excessively high ammonium concentrations were not studied.

Although no link between ammonium and chlorophyll b:a concentrations has been reported to date, it is possible that the increase in chlorophyll b:a ratio with increasing ammonium concentrations may have been indicative of a stress response. Increased chlorophyll b:a ratios have been previously associated with drought (Haisel et al. 2006), salinity (Ranjbarfordoei et al. 2006), heat stress (Georgieva and Lichtenthaler 2006), nutrient deficiency, and nutrient toxicity (Tzvetkova and Hadjiivanova 2006).

At lower ammonium concentrations (A-L, A-M treatments), aspen seedlings were capable of assimilating and utilizing some of the supplied ammonium in support of increased growth and for stomatal and root hydraulic function. Compared with previous papers that describe aspen as an ammonium-sensitive species (Min et al. 2000, Britto and Kronzucker 2002), the results of this study suggest that ammonium may not be toxic to aspen at moderate concentrations such as those provided in the A-L and A-M treatments. Aspen in these treatments (A-L, A-M) fared equally well or slightly better than the nitrate-treated (N-L, N-M) aspen, based on some of the measured parameters (lower chlorophyll b:a ratio, slightly lower E_a, and higher g_s, root:shoot ratio, and L_{pr}). These results were somewhat inconsistent with the study of Guo et al. (2002) who reported decreases in L_{pr} and dry root mass when ammonium was provided as the sole source of nitrogen. It is possible that, partly because of aspen's tendency to exhibit excess, unregulated ammonium uptake due to futile transmembrane ammonium cycling (Min et al. 1999, 2000, Britto et al. 2001), aspen may require less ammonium than nitrate for growth. This may partly explain why aspen in the A-L and A-M treatments had greater growth than aspen in the N-L and N-M treatments. This may also explain how trembling aspen trees are able to survive in mature boreal forests with nitrogen concentrations that were lower than (Vitousek et al. 1982, Balisky et al. 1995, Jerabkova et al. 2006), or similar to (Choi et al. 2007), those used in the present study.

Aspen also appeared to be somewhat capable of responding to moderate increases in ammonium concentration and was capable of utilizing the additional ammonium for growth. A-M-treated aspen had higher g_s, lower root:shoot ratio, and higher L_{pr}, compared to A-L. Although leaf N concentrations in the A-L and A-M treatments were slightly higher than for the N-L and N-M treatments, which may have been due to futile transmembrane cycling (Britto et al. 2001), leaf N was approximately the same between A-L and A-M in spite of the difference in applied ammonium concentrations. This suggests that within this range of moderate ammonium concentrations that were supplied, there did not appear to be an excess accumulation of ammonium or ammonium-derived proteins.

Although aspen may not be as ammonium-sensitive as previously considered, ammonium may have some negative effects even at moderate concentrations. The A-M treatment had higher mortality and a higher chlorophyll b:a ratio compared to the A-L treatment in this study. It is possible that these negative effects may be due to some ammonium sensitivity characteristics within aspen seedlings, which may be genetically determined. Mortality of the A-M treatment was almost twice that of the A-L treatment, yet the surviving aspen seedlings in the A-M treatments generally had increased growth, g_s, and L_{pr} compared to the A-L treatment suggests that there may be some ammonium tolerance variability within aspen. Considering that the aspen used in this study were germinated from seeds, it is possible that genetic variability may have been a factor in aspen's ability to grow in the presence of ammonium.

The form and level of nitrogen supplied did have an effect on the extent of Hg-induced changes in L_{pr}, and PTS_3 concentrations in xylem exudate prior to the addition of $HgCl_2$. The L_{pr} of nitrate-treated aspen was more responsive to Hg, as indicated by a relative decrease in L_{pr} with the addition of $HgCl_2$, and this Hg-responsiveness increased with increasing nitrate concentrations. The increased responsiveness suggests that either a greater proportion of L_{pr} consisted of AQP-mediated transport, or that AQPs in nitrate-treated roots were more sensitive to Hg as a result of the proximity of cysteine residues to the apoplast (Tyerman et al. 1999, Lovisolo and Schubert 2006). It is known that conformational AQP changes can occur in response to environmental conditions, such as pH and anoxia (Tournaire-Roux et al. 2003), resulting in increased or decreased AQP-mediated root water flux. Hg sensitivity has been previously reported in plants that were supplied with high (~18 mM) concentrations of nitrate, compared with lower concentrations (Li and Shao 2003).

Although ammonium-treated aspen seedlings also showed a slight decrease in L_{pr} with the addition of $HgCl_2$, the effect was similar regardless of ammonium concentration. AQPs in ammonium-treated aspen roots were therefore less responsive to $HgCl_2$, possibly because a smaller proportion of L_{pr} was due to AQP-mediated flux, or because AQPs were less Hg-sensitive due to conformational changes. Since it has been shown that nitrogen deficiency can affect lipid membrane composition and therefore affect AQP conformation (Li and Shao 2003), it is possible that the form of nitrogen could affect AQP expression and activity or result in conformational changes that affect AQP function. Decreased AQP expression has been previously observed when ammonium was the only source of nitrogen provided to bean plants (Guo et al. 2002). Further studies would be required to better understand the direct effects of ammonium and nitrate on AQP activity and structure.

PTS_3 concentration prior to the addition of $HgCl_2$ was also affected by form and level of nitrogen in a pattern similar to that of g_s. Although it is possible that transpirational water loss and therefore g_s may have affected the extent of apoplastic root water flux without affecting overall L_{pr} (Carvajal et al. 1996, Li and Shao 2003), there was little correlation between PTS_3 concentrations and g_s for all nitrogen treatments (data not shown). The results of the present study suggest that apoplastic flux, as measured by PTS_3, increased with increasing nitrate concentrations and decreased with increasing ammonium concentrations, respectively. The fact that L_{pr} and PTS_3 were affected somewhat differently by the different nitrogen treatments suggests that the contribution of apoplastic flux to L_{pr} was different for each of the nitrogen treatments. It is possible that the relative importance of apoplastic flux may be related to the extent of AQP-mediated root water flux. However, in the present study, there did not appear to be a direct relationship between changes in apoplastic and AQP-mediated flux because increases in PTS_3 concentration after the addition of $HgCl_2$ were similar for all nitrogen treatments regardless of initial PTS_3 concentrations. This is in contrast to the effect of $HgCl_2$ on AQP-mediated contributions to L_{pr} which differed by nitrogen treatment. It was assumed that if the extent of AQP-mediated root water flux directly affected apoplastic root water flux, then a decrease in L_{pr} following the addition of $HgCl_2$ would result in a corresponding increase in PTS_3 concentration (Siemens and Zwiazek 2003), but this was not observed. These results suggest that apoplastic root water flux may be affected by factors other than AQP activity in roots. Such factors may include changes in root anatomy or biochemical or physiological processes, which may be affected by differences in nitrogen form and level. Environmental stressors, such as aluminum, have previously been shown to inhibit apoplastic flux and root growth without affecting overall root water flux (Sivaguru et al. 2006). Therefore, nitrogen deficiency in the N-L and N-M treatments may have resulted in root physiological changes that affected apoplastic flux without resulting in large changes in L_{pr}. However, the effect of nitrogen on root water flux dynamics is not well understood and should be further studied.

While it is also possible that PTS_3 concentration may not always accurately represent apoplastic flux, due to the molecule's large size and its tendency to enter the xylem through small fissures at lateral root branch junctions (Steudle and Peterson 1998), it is still used and considered to be a measure of relative changes in apoplastic flux (Kumiko and Toru 2002, Siemens and Zwiazek 2003, Schaider et al. 2006).

E_a, a measure of the amount of energy required for the transport of water across cell membranes, was somewhat affected by form of nitrogen. In the present study, mean E_a values ranged between 5.5 – 7 kcal mol^{-1} depending upon the treatment. A low concentration of nitrate resulted in a slight increase in E_a compared to an equivalent concentration of ammonium or to no nitrogen. Previous studies of cell membrane transport have reported that an E_a of $\leq$6 kcal mol^{-1} is indicative of AQP-mediated passive water transport, whereas restriction of water movement through AQPs increases E_a values (Maurel 1997, Shütz and Tyerman 1997). However, these studies measured E_a for cell membranes, not for whole plant tissues or root systems. Because root systems have multiple pathways of resistance to water flux, it is possible that non-AQP-mediated pathways such as water movement through the apoplast, may offer less resistance.

Increased water flux through these low-resistance pathways may result in lower E_a values, which may not be indicative of increased AQP-mediated water flux. Under such conditions, a threshold value of 6 kcal mol^{-1} may be meaningless as an indicator of AQP-mediated water flux at the whole-root level. At least two other studies have reported whole-root E_a values > 6 kcal mol^{-1} (Wan and Zwiazek 1999, Kamaluddin and Zwiazek 2004). Additionally, two previous studies have shown that E_a values were higher in roots with less AQP-mediated water transport, as determined by Hg-induced decreases in root water flux and AQP activity (Wan and Zwiazek 1999, Muhsin and Zwiazek 2002). In the present study, it is possible that the minor differences in E_a between the N-L, A-L, and N-0 treatments may have also been indicative of the extent of apoplastic root water flux, since the N-L treatment had the lowest PTS_3 concentration and the highest E_a, compared with those of A-L and N-0.

2.4.2. Eight-week treatment

In comparing the 4- and 8-week data, these results suggest that there was slight recovery from toxicity in aspen when the highest ammonium concentrations were reduced, although the extent of the changes between the 4- and 8-week treatments varied with the measured parameters and with the initial ammonium concentrations. For many of the measured parameters, reducing ammonium concentrations did not result in any noticeable changes. At low and moderate ammonium concentrations (A-L, A-M), there may have been some evidence of recovery among the leaf-based parameters, as indicated by increased g_s and stem diameter. Although there were some significant changes in root-based parameters at low and moderate ammonium concentrations (increased root:shoot FW ratio, increased PTS_3 concentration, decreased normalized PTS_3 concentration, decreased L_{pr}), it is not possible to conclusively state that such changes were the result of ammonium recovery. Low and moderate ammonium concentrations, as provided during the first four weeks of the experiment, did not appear to result in ammonium toxicity in aspen. Typically, lowered root water flux, decreased AQP-mediated water transport, and changes in root:shoot ratios may be characteristic of nitrogen deficiency (Radin and Matthews 1989, Clarkson et al. 2000, Guo et al. 2007).

Therefore, it is possible that the observed changes in root-based parameters from the reduction in ammonium concentrations (A-L, A-M treatments) were the result of nitrogen deficiency. The fact that shoot-based parameters seemed to exhibit changes consistent with ammonium recovery, as opposed to the changes in root-based parameters, may be because nitrogen translocation to shoots decreases with increasing concentrations of ammonium (Min et al. 1999). Therefore, there may have been a relative increase in ammonium translocation to shoots as a result of the reduction in ammonium concentrations.

With high ammonium concentrations, which appeared to be within the toxic range during the first four weeks, there was some evidence of recovery (increased L_{pr}, decreased mortality, decreased leaf N concentration) with reduction of ammonium concentrations. The decrease in leaf N concentration to the level of the A-L and A-M treatments was expected, and was the only change that was significant. Although the decrease in leaf N may have indicated an elimination of excess ammonium from tissues, it is debatable as to whether or not this reduction could be interpreted as a positive or negative effect of reduced ammonium concentrations. The results suggest that recovery from ammonium toxicity was possible for those seedlings that survived the initial ammonium concentrations. Additionally, the 8-week ammonium concentrations used in the present study were within the range of or lower than ammonium concentrations found in boreal forest soils (Vitousek et al. 1982, Balisky et al. 1995, Jerabkova et al. 2006). The fact that the ammonium concentrations used in the 8-week treatments were within an approximate range of total nitrogen and ammonium concentrations within the boreal forest suggests that aspen is capable of tolerating such ammonium concentrations.

2.4.3. Conclusions

This study demonstrated that aspen did not seem to be as ammonium-sensitive as previously suggested by others (Min et al. 2000, Britto and Kronzucker 2002, Kronzucker et al. 2003). In the present study, low and moderate concentrations of ammonium (4-week concentrations) resulted in increased growth, g_s, and L_{pr} compared to equivalent concentrations of nitrate, suggesting that aspen is capable of utilizing and assimilating ammonium in support of increased growth and physiological function. These results also suggest that aspen may require less ammonium than nitrate for growth, since the low and moderate nitrate concentrations supplied may have resulted in slight nitrogen deficiency, as indicated by several measured parameters. Aspen's growth response to 8-week ammonium concentrations (50% reduction from 4-week concentrations) generally did not differ much from the 4-week measurement parameters, although it is possible that slight ammonium deficiency occurred as a result of the reduction in ammonium concentrations.

However, at high concentrations of nitrate and ammonium (4-week treatment), aspen showed a strong preference for nitrate vs. ammonium, with high ammonium concentrations resulting in toxicity symptoms such as reduced growth, g_s, and L_{pr}, and increased mortality of aspen seedlings. High concentrations of nitrate resulted in substantially higher growth, g_s, and L_{pr} compared to equivalent ammonium concentrations or to lower nitrate concentrations. Aspen, an early-successional species, was expected to show increased growth in the presence of high levels of nitrate, as its preference for nitrate has been previously reported (Min et al. 1998, 1999). The higher nitrogen requirements of aspen suggest that aspen may benefit from nitrate-based fertilization in forest management operations where aspen is being cultivated to maximize growth and productivity, particularly in nutrient-poor soils.

In spite of ammonium toxicity at high concentrations, some recovery from ammonium toxicity was observed (increased L_{pr}, decreased mortality, decreased leaf N concentration) with reduced ammonium concentrations (8-week treatment). Therefore, aspen did demonstrate some ability to recover from ammonium toxicity in the present study. This detail may become important with respect to nitrogen deposition from various anthropogenic sources if the predominant form is present as ammonium, and may be of importance in the remediation of forested areas affected by high ammonium inputs, such as farming and agricultural operations. Such inputs may affect the sources of nitrogen available to tree species, and may become a concern for plant species that exhibit ammonium toxicity symptoms with elevated concentrations of ammonium.

The fact that aspen showed increased growth, g_s, and L_{pr} at the low and moderate concentrations of ammonium used in the present study indicates that aspen should not exhibit reduced growth, photosynthesis, or root hydraulic function, due to either nitrogen deficiency or ammonium toxicity in their natural environment. The 4-week and 8-week concentrations of either nitrate or ammonium used in the L (A-L, N-L) and M (A-M, N-M) treatments are similar to or somewhat higher than those of typical boreal forest soils. Therefore, aspen is likely able to acquire sufficient nitrogen from its ecological environment to support its growth. If insufficient inorganic nitrogen is present, as may be the case in unfertilized and coniferous soils where organic nitrogen concentrations are higher (Ferrari 1999, Andersson and Berggren 2005), it is possible that aspen, as well as mycorrhizal fungi associated with aspen roots, may be able to acquire and assimilate organic forms of nitrogen from soil (Näsholm et al. 1998, Persson and Näsholm 2001).

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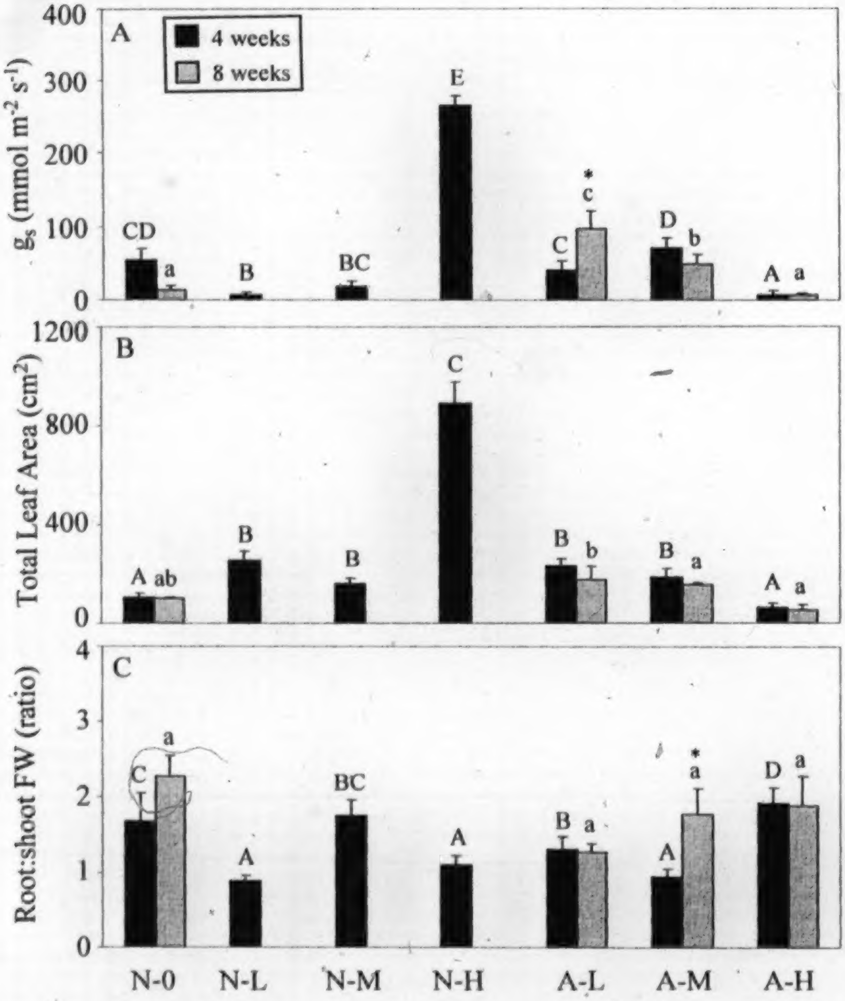

Figure 2.1. (**A**) Stomatal conductance (g_s), (**B**) total leaf area, and (**C**) root:shoot fresh weight ratio, in aspen seedlings receiving either no nitrogen (N-0), or one of the following nitrogen treatments for either 4 or 8 weeks: low nitrate (N-L), medium nitrate (N-M), high nitrate (N-H), low ammonium (A-L), medium ammonium (A-M), or high ammonium (A-H). 8-week treatments (N-0, A-L, A-M, and A-H) consisted of ammonium concentrations that were 50% less than 4-week treatments. Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments for 4-week treatments), lowercase letters (among nitrogen treatments for 8-week treatments), and asterisks (between 4- and 8-week treatments for each nitrogen treatment).

Table 2.1. ANOVA table indicating p-values of main effects and interactions for 4-week measurements of response variables to nitrogen form (nitrate, ammonium) and level (low, medium, and high) treatment combinations in aspen seedlings. Only response variables with at least one significant ($p\leq 0.05$) effect are shown. Effects were tested for significance at $\alpha=0.05$ using PROC MIXED (SAS 9.1). Slice operation (by nitrogen form) results from SAS are also shown as superscripts beside significant ($p<0.05$) interaction p-values: ^{N} = sig. differences between levels for nitrate-only treatments, ^{A} = sig. differences between levels for ammonium-only treatments.

			Response Variables			
Effect	g_s	Root:Shoot FW	Stem Diameter	Leaf Area	Leaf Total Nitrogen	Shoot Ψ_w
Nitrogen Form	p<0.0001	0.07667	0.4389	p<0.0001	0.1833	0.95
Level	p<0.0001	p<0.01	p<0.05	p<0.0001	p<0.01	0.076
Nitrogen*Level	p<0.0001$^{N A}$	p<0.0001$^{N A}$	p<0.0001$^{N A}$	p<0.0001$^{N A}$	p<0.05$^{N A}$	p<0.001^{A}

			Response Variables			
Effect	Total Chlorophyll	Chl. b:a	L_{pr}	Normalized L_{pr}	$[PTS_3]$	Normalized $[PTS_3]$
Nitrogen Form	p<0.01	p<0.01	0.6571	0.8656	0.3395	0.2312
Level	p<0.0001	0.07211	0.5536	p<0.05	0.2053	p<0.01
Nitrogen*Level	p<0.0001^{N}	p<0.05^{A}	p<0.0001$^{N A}$	0.06811^{N}	p<0.01^{N}	0.174561^{N}

Table 2.2. Shoot water potential (Ψ_w), stem diameter (Stem Dia.), and total chlorophyll (Tot. Chl.) in aspen seedlings receiving either no nitrogen (N-0), or one of the following nitrogen treatments for either 4 or 8 weeks: low nitrate (N-L), medium nitrate (N-M), high nitrate (N-H), low ammonium (A-L), medium ammonium (A-M), or high ammonium (A-H). 8-week treatments (N-0, A-L, A-M, and A-H) consisted of ammonium concentrations that were 50% less than 4-week treatments. Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by lowercase letters (between nitrogen treatments within either 4-week or 8-week groups) and asterisks (between 4- and 8-week groups for the same nitrogen treatment).

4-week treatments

Measurements	N-0	N-L	N-M	N-H	A-L	A-M	A-H
Shoot Ψ_w (MPa)	-0.56±0.068a	-0.94±0.12ab	-1.06±0.24bc	-0.72±0.13ab	-0.58±0.053a	-0.64±0.074a	-1.47±0.18c
Stem Dia. (mm)	4.5±0.23ab	4.0±0.21a	4.4±0.22ab	5.6±0.33d	4.8±0.089bc	4.4±0.15ab	4.2±0.19ab
Tot. Chl. (mg g^{-1} FW)	1.55±0.15ab	1.04±0.15a	1.95±0.072bc	3.23±0.28bc	1.75±0.19b	1.69±0.17b	1.53±0.28a

8-week treatments

Measurements	N-0	A-L	A-M	A-H
Shoot Ψ_w (MPa)	-0.90±0.058ab	-0.80±0.069ab	-0.67±0.046a	-1.30±0.29b
Stem Dia (mm)	4.6±0.30a	5.2±0.39a	5.2±0.084a*	4.5±0.21a
Tot. Chl. (mg g^{-1} FW)	0.87 ± 0.32a	1.84 ± 0.22b	1.89 ± 0.19b	1.46±0.21ab

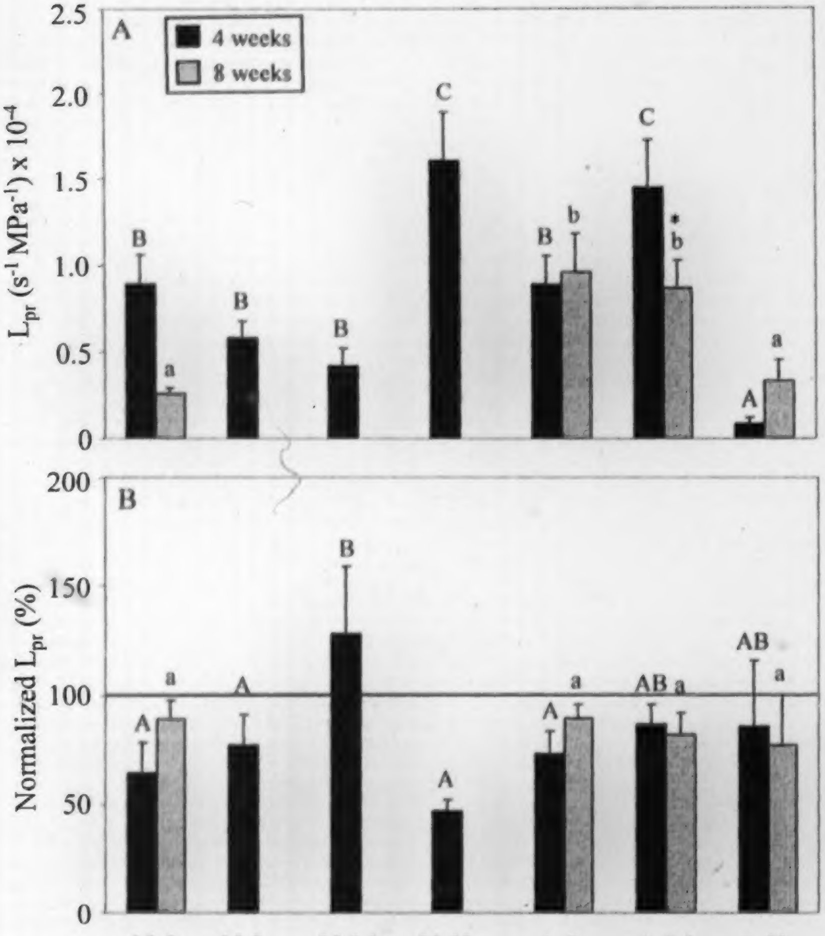

Figure 2.2. (**A**) Root hydraulic conductivity (L_{pr}) measured prior to treatment of roots with $HgCl_2$. (**B**) Normalized L_{pr} measured after the addition of 50 μM $HgCl_2$ and calculated as a percentage of untreated L_{pr} values (shown in **Fig. 2.2A**, and indicated by the line at 100% in **Fig. 2.2B**). Aspen seedlings received either no nitrogen (N-0), or one of the following nitrogen treatments for either 4 or 8 weeks: low nitrate (N-L), medium nitrate (N-M), high nitrate (N-H), low ammonium (A-L), medium ammonium (A-M), or high ammonium (A-H). 8-week treatments (N-0, A-L, A-M, and A-H) consisted of ammonium concentrations that were 50% less than 4-week treatments. Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments for 4-week treatments), lowercase letters (among nitrogen treatments for 8-week treatments), and asterisks (between 4- and 8-week treatments for each nitrogen treatment).

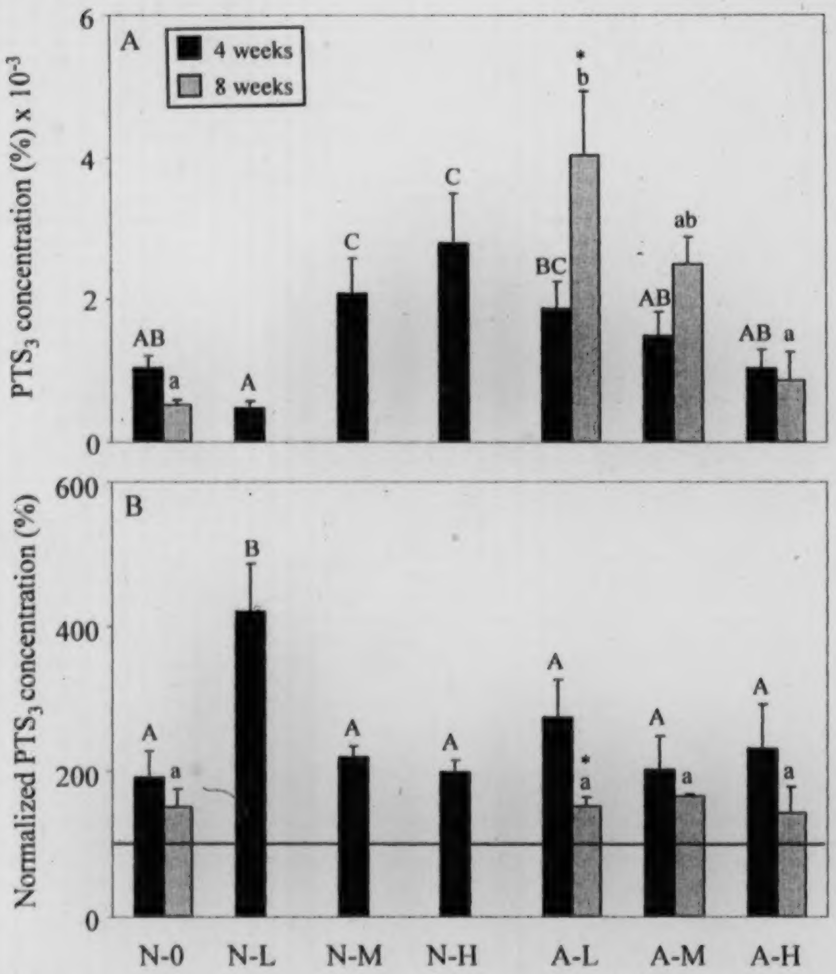

Figure 2.3. (**A**) PTS$_3$ concentration in xylem exudate of roots prior to treatment with HgCl$_2$. (**B**) Normalized PTS$_3$ concentration measured after the addition of 50 µM HgCl$_2$ and calculated as a percentage of untreated PTS$_3$ values (shown in **Fig. 2.3A**, indicated by the line at 100% in **Fig. 2.3B**). Aspen seedlings received either no nitrogen (N-0), or one of the following nitrogen treatments for either 4 or 8 weeks: low nitrate (N-L), medium nitrate (N-M), high nitrate (N-H), low ammonium (A-L), medium ammonium (A-M), or high ammonium (A-H). 8-week treatments (N-0, A-L, A-M, and A-H) consisted of ammonium concentrations that were 50% less than 4-week treatments. Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments for 4-week treatments), lowercase letters (among nitrogen treatments for 8-week treatments), and asterisks (between 4- and 8-week treatments for each nitrogen treatment).

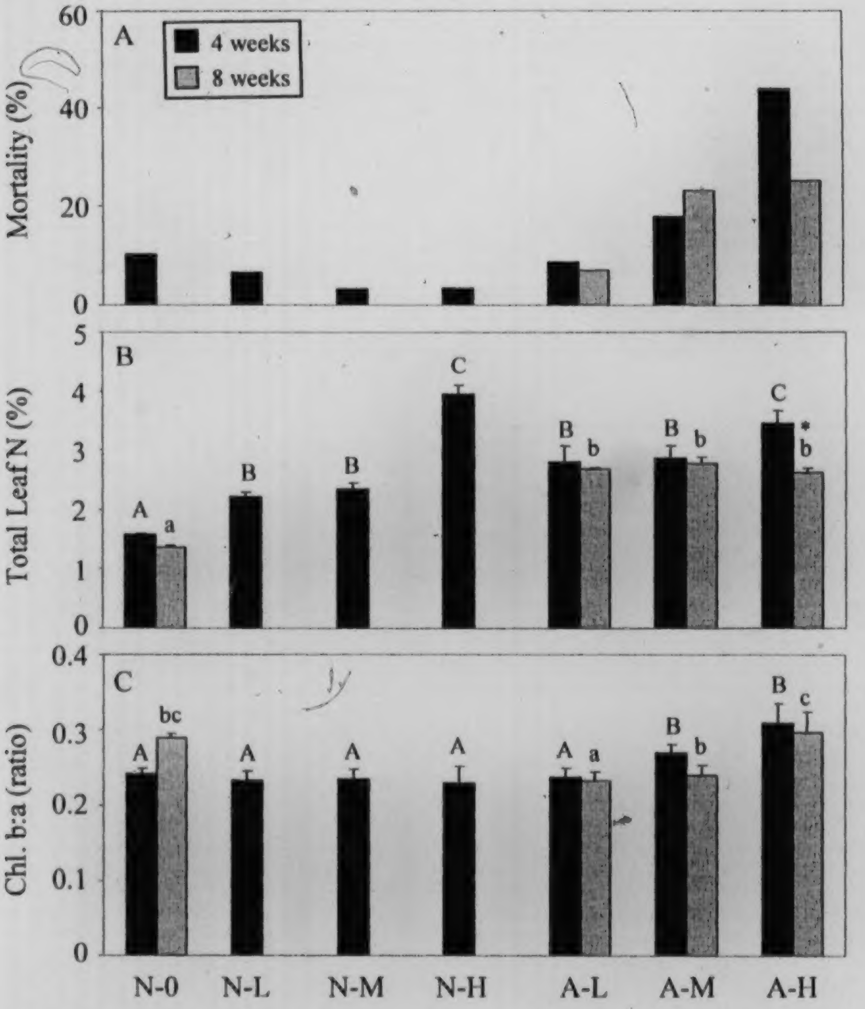

Figure 2.4. (**A**) percent mortality, (**B**) total leaf nitrogen, and (**C**) chlorophyll b:a ratio, in aspen seedlings receiving either no nitrogen (N-0), or one of the following nitrogen treatments for either 4 or 8 weeks: low nitrate (N-L), medium nitrate (N-M), high nitrate (N-H), low ammonium (A-L), medium ammonium (A-M), or high ammonium (A-H). 8-week treatments (N-0, A-L, A-M, and A-H) consisted of ammonium concentrations that were 50% less than 4-week treatments. Single values are shown for **Fig. 2.4A** (n=36). Least-squares means for **Fig. 2.4B,C** ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments for 4-week treatments), lowercase letters (among nitrogen treatments for 8-week treatments), and asterisks (between 4- and 8-week treatments for each nitrogen treatment).

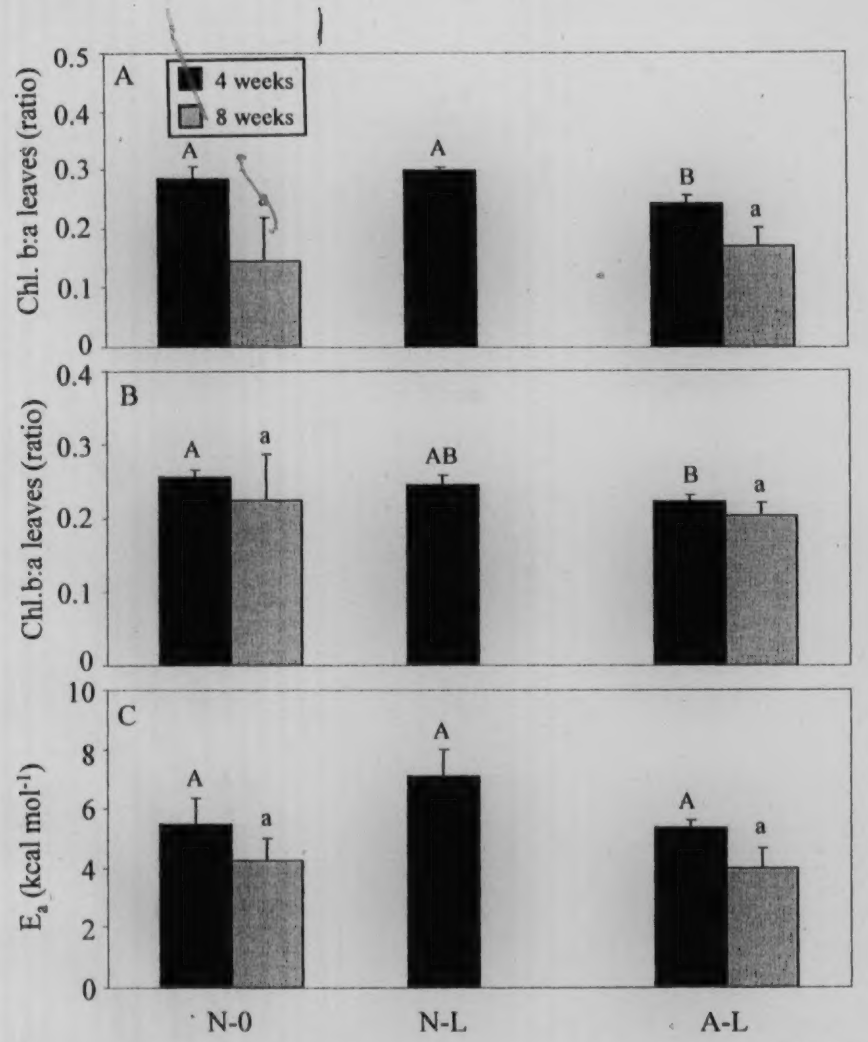

Figure 2.5. Leaf chlorophyll b:a ratios from the upper (**A**) and lower (**B**) part of the shoot, and (**C**) the activation energy (E_a) of root water transport, in aspen seedlings receiving either no nitrogen (N-0), or one of the following nitrogen treatments for either 4 or 8 weeks: low nitrate (N-L), or low ammonium (A-L). 8-week treatments (N-0, A-L) consisted of ammonium concentrations that were 50% less than 4-week treatments. Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments for 4-week treatments), lowercase letters (among nitrogen treatments for 8-week treatments), and asterisks (between 4- and 8-week treatments for each nitrogen treatment).

CHAPTER III

Growth, physiology, and nitrogen metabolism in mycorrhizal trembling aspen seedlings subjected to nitrate and ammonium treatments, and nitrogen metabolism in *Hebeloma crustuliniforme* mycelia.

3.1. INTRODUCTION

Trembling aspen and other species of *Populus*, including hybrid poplars, are of commercial interest because of their fast growth in intensively managed forests (Rytter and Stener 2003) and their use in pulp, paper, and oriented strand board products (Peterson and Peterson 1992). Since nitrogen nutrition can affect growth (DesRochers et al. 2006) and secondary xylem fiber properties (Pitre et al. 2007), understanding the long-term effects of type and level of nitrogen applications in forest management are of great importance.

In mature boreal forest soils, the form of inorganic nitrogen available to plants can be affected by factors such as pH and temperature (Bloom 1988), with ammonium generally being the predominant form of inorganic nitrogen available to and utilized by many tree species (Nordin et al. 2001, Titus et al. 2006). Many boreal tree species, especially conifers, show preferential uptake and assimilation of ammonium over nitrate (Kronzucker et al. 1997, 2003) and increased growth when ammonium is supplied in greenhouse studies instead of nitrate (Lavoie et al. 1992). Other tree species within the boreal forest, such as trembling aspen and Douglas-fir, show a strong preference for nitrate. It has been suggested that these species are ammonium-sensitive because previous studies have reported increased influx and accumulation of ammonium when it was supplied at concentrations equivalent to those found in native boreal soils (Min et al. 1999, Kronzucker et al. 2003). It has been hypothesized that preference for a particular form of inorganic nitrogen is related to the ecological roles of trees in forest succession. Late-successional species prefer ammonium, which is abundant in late-successional soils, and early-successional tree species demonstrate a preference for nitrate, which is abundant in early-successional forest soils and is initially present in disturbed soils (Prescott 1997, Min et al. 1998, Jerabkova and Prescott 2007). However, this theory is not always supported by ecological observations. Fraser fir (*Abies fraseri*), a climax species, has been reported to prefer nitrate over ammonium (Rothstein and Cregg 2005). A recent study has also reported that soil nitrate levels are not higher in aspen-dominated boreal forests than in conifer-dominated forests, and that clear-cutting disturbance does not necessarily result in increased soil nitrate concentrations (Jerabkova and Prescott 2007).

Ammonium-sensitivity in some plant species has been explained by the theory of futile transmembrane ammonium cycling, resulting in excessive ammonium accumulation in plant tissues (Britto et al. 2001). In spite of understanding how and why ammonium may be toxic to tree species that have been described as ammonium-sensitive, there has been little research to explain how these tree species are capable of surviving and growing to maturity in mature boreal forests where soil ammonium concentrations are often high and where soil nitrate concentrations are low or non-existent (Jerabkova et al. 2006). However, some studies suggest that addition of nitrate may ameliorate symptoms of ammonium toxicity in crop species (Cruz et al. 1993, Schortemeyer et al. 1997), and that the ratio of ammonium to nitrate is important for tree growth (Stadler et al. 1993, Rothstein and Cregg 2005).

It is possible that ectomycorrhizal (ECM) fungi associated with tree species that prefer nitrate may be capable of increasing their plant hosts' ammonium tolerance. In previous studies, ECM fungi have been observed to mitigate the effects of toxic substances such as heavy metals and salts (Jentschke and Godbold 2000, Kernaghan et al. 2002). ECM fungi have been reported to increase nutrient availability by exploiting greater soil volumes and more distant sources of nutrients through their hyphal projections from roots (Mukerji et al. 2000, Hodge 2003). Fungal enzymatic breakdown of organic soil compounds can also increase nutrient availability to plants in the surrounding soil (Dexheimer et al. 1986, Tibbett et al. 1998, Finlay et al. 2002) and assist in selective uptake of nitrate and potassium ions (Plassard et al. 2002). Therefore, it is possible that ECM fungi may be capable of providing localized deposits of nitrate which are readily taken up by hyphae, or may divert excess ammonium into fungal tissue for storage or for conversion to other forms of nitrogen that can be transferred to the plant host.

Although the role of ECM fungi in the nitrogen metabolism of ammonium-sensitive tree species has not been studied, it can be hypothesized that ECM fungi may physiologically alter their plant hosts' tolerance by altering enzymatic processes involved in nitrogen metabolism. There is little information regarding the actual physiological control of ammonium assimilation in plants (Oaks and Hirel 1985). In some temperate plant species, inorganic nitrogen is assimilated directly in roots (Scheromm and Plassard 1988) via nitrate reductase, which catalyzes the first step in nitrate assimilation (Oaks 1994), and via glutamine synthetase (GS) and glutamate synthase (GOGAT) that catalyze the first steps in ammonium assimilation (Lea et al. 1992, reviewed in Joy 1988). Another enzyme, glutamate dehydrogenase (GDH), which is thought to be negligibly involved in plant ammonium assimilation (Magalhães et al. 1990, Joy 1988), may be primarily involved in ammonium metabolism in ECM fungi (Ahmad and Hellebust 1991, Chalot et al. 1990b). It is possible that ECM fungi-plant root interactions may alter or suppress plant or fungal enzymatic processes via biochemical or structural changes (Botton and Dell 1994, Chalot et al. 1990a). However, it is also possible that mycorrhizal associations may enhance the activity of GS and other enzymes involved in nitrogen metabolism in plant host tissues (Botton et al. 1989, Cliquet and Steward 1993, Blaudez et al. 1998).

The objectives of this study were: 1) to examine growth, xylem sap properties, nitrogen-metabolizing enzymatic activity, and ^{15}N uptake in mycorrhizal and non-mycorrhizal trembling aspen grown with low or high concentrations of nitrate, ammonium, and a combination of nitrate and ammonium; and 2) to examine growth and nitrogen-metabolizing enzymatic properties of the same mycorrhizal fungus, *Hebeloma crustuliniforme*, grown on media treated with equivalent forms and concentrations of nitrogen. It was predicted that mycorrhizal seedlings, compared to non-mycorrhizal seedlings, would show increased growth when treated with higher levels of nitrogen, particularly in the nitrate treatments. It was also predicted that most of the nitrogen taken up by plants would be assimilated in the roots, as indicated by xylem sap composition, and that the presence of *H. crustuliniforme* would increase aspen's ammonium tolerance and the activity of ammonium assimilating enzymes in ECM root tissues.

3.2. MATERIALS AND METHODS

3.2.1. Mycorrhizal culture

Hebeloma crustuliniforme (Bull.) Quél. (University of Alberta, Devonian Microfungus Collection, UAMH 5247) was sub-cultured on Melin Norkrans Media (MNM) agar (Mason 1980). Plugs of MNM-grown fungus were aseptically transferred to MNM liquid media in aerated flasks, and placed on an orbital shaker for 4 weeks to produce liquid culture. Mycelia grown in liquid culture was homogenized under sterile conditions using a blender to produce liquid inoculum for seedling roots.

Plugs of MNM-grown fungus were sub-cultured on prepared plates with Murashige and Skoog (MS) agar media (Murashige and Skoog 1962), modified by decreasing the sucrose concentration (3 g L^{-1}) to create a low-sucrose media for pure fungal culture. MS media, although typically used for growth of plant cell cultures, was used in this experiment since mycorrhizal seedlings inoculated with *H. crustuliniforme* had been successfully cultivated in aseptic culture using low-sucrose MS media in a previous pilot study. *H. crustuliniforme* was grown for approximately 4 weeks on low-sucrose MS agar, prior to transfer to nitrogen treatment plates in preparation for enzyme assays of mycelial tissue.

3.2.2. Plant culture

Trembling aspen (*Populus tremuloides* Michx.) seedlings were grown from seed collected from the North Saskatchewan river valley (Edmonton, Alberta, Canada) in 2004 and stored at -10°C until use. Seeds were removed from storage and germinated on washed, sterile silica sand for 4 days, then transferred to styroblocks™ (superblock 160/60, Beaver Plastics Ltd., Edmonton, AB, Canada) containing washed, sterile silica sand. Trays were placed in a controlled-environment growth chamber (60% RH, 18 hr photoperiod, 22°C/18°C day/night, 300 μmol m^{-2} s^{-1} PAR, full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada)). Trays were bottom-watered every other day with deionized water, and once weekly with 0.1% 20-20-20 fertilizer (Plant Prod® Water-Soluble Fertilizer, Spectrum Brands Inc.) for 4-5 weeks. Sources of nitrogen used in this 20-20-20 fertilizer formulation were not available.

During weeks 5-7, fertilizer treatment was eliminated prior to mycorrhizal inoculation of seedling roots.

3.2.3. Mycorrhizal inoculation of aspen seedlings

Seedlings were removed from styroblocks™, and roots were gently rinsed with deionized water to remove excess sand. For half of the seedlings, 5 mL of homogenized mycelia was directly applied to each rinsed root system with a wide-tip sterile plastic pipette. Inoculated seedlings were transferred to clean 75 mm pots which had drainage holes at the bottom. Pots were lined with a thin layer of cheesecloth and filled with washed, sterile silica sand. The remaining seedlings were designated as non-mycorrhizal control seedlings, "inoculated" with 5 mL of autoclaved fungal-free liquid MNM, and transplanted in the same manner as the mycorrhizal seedlings. Pots of mycorrhizal (MYCO) and non-mycorrhizal (CTRL) seedlings were kept physically separated from each other by placing them in leak-proof plastic tubs (1 tub per nitrogen treatment). These tubs prevented any leakage of watering solution from the pots onto the floor, and sides were high enough to prevent any splashing of nearby pots during watering, thereby preventing contamination of CTRL seedlings from the fungal inoculum introduced to MYCO seedlings. Seedlings were top-watered with a small amount of deionized water every other day for weeks 8-9. During week 10, bottom-watering of seedlings began. Seedlings were watered every other day with deionized water. During week 11, 0.025% 20-20-20 fertilizer solution was supplied once per week instead of water according to their current watering schedule. During week 12, the concentration of once-weekly 20-20-20 fertilizer solution was increased to 0.05% and remained at this concentration until nitrogen treatments began. Seedlings were grown for a total of 13-17 weeks prior to introduction of nitrogen treatments.

3.2.4. Nitrogen treatments for aspen seedlings

Three different treatments of inorganic nitrogen (nitrate only, N; ammonium only, A; nitrate + ammonium, NA) and two different concentrations (low, 2 mM total nitrogen; high, 8 mM total nitrogen), for a total of six different nutrient treatment combinations, (nitrogen form * concentration) were applied to MYCO and CTRL seedlings. The 2 mM and 8 mM concentrations were selected because the former concentration represents typical nitrogen concentrations found in some boreal forest soils, whereas the latter concentration is higher than typical nitrogen concentrations found in boreal forest soils. Additionally, a 0 mM no nitrogen (O) treatment was applied to MYCO and CTRL seedlings as a negative nitrogen control. The nutrient solutions for all treatments were created from modified 100% Hoagland's solution (Hoagland and Arnon 1950), and adjusted to pH 5.5. Nutrient solutions for all treatments contained concentrations of minerals other than nitrogen that were equal to or greater than the formula for 100% Hoagland's solution to prevent nutrient deficiency, but were insufficient to induce toxicity. Concentrations of nutrients other than nitrogen were as follows: O (K=6 mM, PO_4=3 mM, SO_4=3.5 mM, Ca=4 mM, Mg=2mM); N (K=6 mM, PO_4=3 mM, SO_4=3.5 mM, Ca=4 mM, Mg=2mM); A-2mM (K=6 mM, PO_4=3.5 mM, SO_4=4 mM, Ca=4 mM, Mg=2mM); A-8mM (K=5.5 mM, PO_4=4.5 mM, SO_4=5.5 mM, Ca=4 mM, Mg=2mM); NA (K=6 mM, PO_4=3 mM, SO_4=3.5 mM, Ca=4 mM, Mg=2mM). Concentrations of micronutrients were the same as for 100% modified Hoagland's solution, and were supplied in mg L^{-1} as follows: Fe-EDTA (5), B (0.5), Mn (0.5), Zn (0.05), Cu (0.02), Mo (0.01). Nitrogen treatments were applied for two months prior to the first set of measurements.

3.2.5. Nitrogen treatments for H. crustuliniforme plate culture

Four nitrogen treatments including no inorganic nitrogen (O), nitrate only (N), ammonium only (A), and nitrate + ammonium (NA) were used for treatment of *H. crustuliniforme* (Hc) in sterile culture. Total inorganic nitrogen concentration was 8 mM in the N, A, and NA treatments. The form of inorganic nitrogen used for the treatments was supplied as $Ca(NO_3)_2$ (N treatment), or $(NH_4)_2SO_4 + (NH_4)_2HPO_4$ (A treatment), or NH_4NO_3 (NA treatment).

Petri plates with low-sucrose modified MS media (Murashige and Skoog 1962) were prepared to produce agar growth media for each of the nitrogen treatments. To control all unquantifiable forms of available carbon and nitrogen in MS media, casein enzyme hydrolysate was omitted from the preparation, although the small concentration of organic N-containing vitamins (nicotinic acid, thiamine-HCl, pyridoxine-HCl) that are part of the MS formula were added to the media. Low sucrose concentrations were supplied (4.7 g L^{-1} for N, A, and NA treatments) to ensure an optimal 20:1 C:N ratio for all inorganic nitrogen-containing treatments. A minimal amount of sucrose (0.12 g L^{-1} for O treatment) was supplied to provide a minimal amount of sucrose in the absence of inorganic nitrogen, resulting in a C:N ratio to 37:1. Purified agar (cat. # A1296, Sigma-Aldrich Canada) was used as the gelling agent for all plates. Although there was no available report for trace element concentration of nitrogen in the purified agar used, it was assumed that it was low (<1%) based on typical trace concentrations of other macronutrients. The media for each treatment were pH-adjusted to ensure optimal growth. In a previous experiment (Chapter IV), it was observed that maximum fungal growth occurred at pH 6 on N, A, and NA media, and at pH 5 on O media.

Because the pH of media changes during autoclaving, and because agar media solidification varies with pH, preliminary experiments were conducted to determine the pH adjustments and quantity of agar that needed to be added to each treatment prior to autoclaving. In order to obtain the correct pH after autoclaving and media solidification, and to make sure that the media in each treatment showed equivalent solidification, 15 g L^{-1} agar was added to N, A, and NA media treatments (pH 6), and 18 g L^{-1} was added to O media (pH 5). All pH measurements were taken with a flat-surface electrode (Cat. # 13620289, Fisher Scientific Co., Ottawa, Canada). Pre- and approximate post-autoclaving pH values for the four nitrogen treatments (O, N, A, NA) are listed below.

Target pH	pH before autoclaving	pH after autoclaving
5	5.10^{O}, 5.05^{N}, 5.14^{A}, 5.13^{NA}	5.10^{O}, 4.83^{N}, 4.94^{A}, 4.98^{NA}
6	6.60^{O}, 7.20^{N}, 6.75^{A}, 6.53^{NA}	6.25^{O}, 5.82^{N}, 6.08^{A}, 6.08^{NA}

A minimum of eight (n=8) 100 mm diameter Petri dishes containing approximately 30 mL each of autoclaved, modified low-sucrose MS media were poured for each nitrogen treatment. Pieces of porous cellophane (Cat. # 361004007, Fisher Scientific Co., Ottawa, Canada) were trimmed, autoclaved separately, and aseptically transferred to the autoclaved, solidified plates of media. Cellophane circles were laid on top of the solidified media of each dish to facilitate harvesting of mycelial tissue at the end of the experiment. One 8 mm dia. plug of low-sucrose MS-grown *H. crustuliniforme*, cut from the periphery of an existing colony, was aseptically transferred to each nitrogen treatment dish and centered in the middle of each piece of cellophane. Dishes were then sealed with Parafilm and stored upright. Two days after completion of mycelial transfers to all nitrogen treatments, all dishes were inverted and incubated at room temperature for 4 weeks to provide sufficient mycelial material for enzyme assays.

3.2.6. Aspen seedling measurements

3.2.6.1. Shoot height, stem diameter and leaf chlorophyll

Shoot height measurements were taken in intact plants, from the base of the stem to the shoot tip. Stem diameter measurements were taken with calipers at the base of the stem.

Leaves from the upper half of the stem were randomly selected for chlorophyll analysis. Several leaves from each plant were immediately frozen in liquid nitrogen, and stored at -80 C. Approximately 0.1 g of frozen leaf tissue from each plant for each treatment was weighed and used for chlorophyll extraction and analysis with DMSO (Hiscox and Israelstam 1979).

3.2.6.2. Leaf area and dry weight

Projected leaf area measurements were taken for all the leaves from each plant by removing the leaves and measuring their total surface area with a LI-3100C leaf area meter (LI-COR, Lincoln, NE, USA). Leaves from each seedling were placed in separate paper bags, dried for 72 hrs at 65°C in a drying oven (Despatch PBC 224, Despatch Industries, Inc., Minneapolis, MN, USA), and leaf dry weights were determined.

3.2.6.3. Sand leachate and xylem exudate measurements

Potted seedlings were removed from the plastic tubs, and the bottoms of the pots were well-rinsed and dried. Each pot was placed in a clean, deep Petri dish to capture leachate, and then each pot was top-watered with approximately 20 mL of deionized water and allowed to fully drain for 20 minutes. Sand leachate was collected from each Petri dish and measured for pH. The pH of the deionized water (mean pH 7.02 ± 0.42) applied to the pots was also recorded for comparative purposes.

Following leachate collection, seedlings were removed from pots with potting sand intact, and placed in plastic bags to tightly contain the root and surrounding sand. Since there was insufficient root pressure in excised root systems to collect xylem sap, root systems were detopped with approximately 3 cm of stem remaining, and placed in Scholander pressure chambers (PMS Instruments, Corvallis, OR). Stem segments protruding from the pressure chamber lids were peeled to remove bark and phloem from the area surrounding the cut surface, and the remaining xylem was gently rinsed with deionized water and blotted dry with KimWipes™ (Kimberley-Clark Corp., UK). Increasing pressure was applied to roots in the pressure chambers until xylem sap appeared at the cut surface. The pressure at which the xylem sap appeared was referred to as the balance pressure. Then, pressure was increased to approximately 0.4 MPa above the balance pressure for each root system to ensure that adequate quantities of xylem exudate were collected within a relatively short (< 30 minutes) time period. It has been previously reported that applied pressure up to 1 MPa above the balance pressure did not affect xylem sap composition (Osonubi et al. 1988). The first drop of xylem exudate was wiped away and discarded to remove potential contaminants from the excision process. Final pressure needed to express xylem exudate from roots was treatment- and seedling-dependent, and varied between 0.6-1.3 MPa. New, clean pieces of flexible silicon tubing were fitted over the cut stem after the first drop of exudate was removed, and the other end was inserted into a new Eppendorf microfuge tube for collection. Once the tubing was connected to the cut stem segment, xylem exudate was collected for 20 minutes, which is considered to be within the range of an acceptable length of time for sampling without the occurrence of substantial changes in xylem sap composition (Husted et al. 2000, Wan et al. 2004). Samples were stored at -20°C until they could be analyzed.

Xylem exudate samples were measured for pH, and for concentrations of nitrate and ammonium ions with a Technicon Autoanalyzer (Technicon Industrial Systems 1973a,b).

3.2.6.4. Root volume and root length colonization

Root systems were gently rinsed to remove potting sand and blotted with paper towels to remove excess water from root surfaces. Root volumes were measured for each root system using volume displacement of water in a graduated cylinder (Voicu and Zwiazek 2004).

To measure root colonization, rinsed whole root systems of selected seedlings were preserved for mycorrhizal analysis in 4:3:3 ethanol-glycerol-water solution, and stored at 4°C. A subsample of roots from each treatment was collected for root colonization measurements. Root systems were removed from preserving solution and dissected to remove all fine roots with a diameter $\leq$0.05 mm. These fine roots were randomly cut into 1-2 cm pieces and spread out in a large, water-filled Petri dish. Approximately 25% of these fine roots were removed from the batch and stored in sealed conical centrifuge tubes filled with 4:3:3 ethanol-glycerol-water solution.

Staining of mycorrhizal roots was conducted similarly to the procedure outlined by Brundrett et al. (1996). Up to half of the stored roots were removed, rinsed well with deionized water on a fine mesh filter, blotted to remove excess water, and placed in glass scintillation vials up to 1/3 full with 10% KOH. Root samples were autoclaved for 20 minutes. When vials were no longer hot to the touch, roots were removed from KOH solution, rinsed well with deionized water on a fine mesh filter, blotted to remove excess water, and placed in scintillation vials containing 0.05% trypan blue-lactoglycerol staining solution, and autoclaved for 12 minutes. When trypan blue solution reached room temperature, solution was carefully pipetted from the scintillation vials, and roots were gently washed several times in the vials, first with deionized water, and then with lactoglycerol to remove excess stain. Stained roots were then stored in lactoglycerol for five days to ensure adequate destaining of roots.

Destained roots were spread out in their lactoglycerol solution in plastic Petri dishes etched with a 4 x 4 - 1 cm^2 grid (FisherBrand Contact Plate, Cat. # 08-757-11C, Fisher Scientific Co., Ottawa, Canada). Dark- and light-stained root segments intersecting the horizontal and vertical lines of the grid were counted to calculate percent root length colonization as described in Brundrett et al. (1996).

3.2.6.5. Enzyme assays

All chemicals for enzyme assays were purchased from Sigma-Aldrich (Canada). MYCO and CTRL seedlings from the 8 mM N, A, NA, and O treatments, were selected for enzyme assays. Leaves and root samples were weighed, rinsed and blotted dry with sterile, Milli-Q water. Leaf and root samples were prepared and nitrate reductase (NR) and glutamine synthetase (GS) assays were conducted according to the methods described by Truax et al. (1994), with 0.2 g L^{-1} chloramphenicol added to the NR root incubation solution to inhibit bacterial nitrate reductase activity. Standard curves for NR and GS were prepared using known concentrations of KNO_2 (A_{540} nm) and L-glutamine-γ-hydroxamate (A_{540} nm), respectively.

NAD- and NADP-glutamate dehydrogenase (GDH) assays were conducted using root tissue according to the preparation and assay methods outlined by Rudawska et al. (1994), using 0.5 g root tissue per sample ground to powder in a mortar with liquid nitrogen. Assays for NAD-GDH and NADP-GDH were performed using the same root extract for both assays, but with different assay buffers (Rudawska et al. 1994) that contained the same molar concentrations of either NADH or NADPH as an enzyme substrate.

Root and leaf extracts used in the enzyme assays were quantified for protein concentration using Peterson's modification of the Lowry protocol (Total Protein Kit, cat.

TP0300, Sigma-Aldrich, Canada) (Peterson 1977) using bovine serum albumin as a standard (A_{550} nm). NR activity was calculated as nmol NO_2 produced h^{-1} mg^{-1} protein. GS activity was calculated as mmol monohydroxamate produced h^{-1} mg^{-1} protein. GDH activity was calculated as nkat mg^{-1} protein (nkat = nmoles NAD(P)H consumed or produced s^{-1}).

3.2.6.6. ^{15}N applications and measurements

MYCO and CTRL seedlings from the 8 mM N, A, NA, and O treatments, were selected for ^{15}N uptake measurements, with twice as many NA seedlings selected compared to the number of selected N, A, and O seedlings. To examine ^{15}N uptake of either nitrate or ammonium when they were supplied together, NA seedlings were randomly chosen to receive either ^{15}N-labeled nitrate or ^{15}N-labeled ammonium forms of NH_4NO_3. Selected seedlings were watered only with deionized water for two weeks prior to application of ^{15}N-enriched solutions. Pots were then removed from the plastic tubs and each placed in the bottom half of a deep Petri dish to catch any flow-through of ^{15}N-labeled solution. Based on a previous pilot experiment using 8 mM ^{15}N nitrogen concentrations, it was decided that seedlings would be harvested 72 h following ^{15}N treatment application.

All ^{15}N isotope chemicals were obtained from Sigma-Aldrich (Canada). A total of four ^{15}N treatment solutions were prepared using the same concentrations of nutrients as for the 8 mM N, A, and NA treatments (described in section 3.2.4.), with 98% atom ^{15}N-labeled stable isotopes substituted for the non-isotopic forms of nitrogen previously used. The ^{15}N forms of nitrogen used were $Ca(^{15}NO_3)_2$ (N treatment), $(^{15}NH_4)_2SO_4$ (A treatment), $NH_4{}^{15}NO_3$ (*NA treatment), and $^{15}NH_4NO_3$ (N*A treatment). Treatment solutions were applied to the N, A, and NA seedlings, with half the NA seedlings receiving the *NA solution, and the other half receiving the N*A solution. Each seedling was top-watered with 20 mL of the appropriate ^{15}N-enriched solutions. Seedlings from the O treatment were re-watered with their original nitrogen-free nutrient solution. Any flow-through captured by the underlying Petri dish was added back into the pot. After 48 h, seedlings were watered with a small (15 mL) quantity of water to maintain adequate soil moisture, and any flow-through captured in the Petri dish was added back into the pot. After 72 h, all seedlings were removed from their pots, and leaves and roots were gently washed with deionized water and blotted to remove excess water. Seedlings were separated into leaves and roots, leaf and root samples were packaged separately in aluminum foil, and immediately frozen in liquid nitrogen for storage at -80°C.

Leaf and root tissue samples from all seedlings were removed from the freezer, placed in individual paper bags, and oven-dried (Despatch PBC 224, Despatch Industries, Inc., Minneapolis, MN, USA) at 50°C for 72 h. All root and leaf tissue was ground in a Wiley mill until samples could be passed through a 20 mesh sieve, then pulverized in a vibrating-ball mill (Retsch, Type MM2, Brinkman Instruments Co., Toronto, Canada). Ethanol, in addition to vaccum cleaning, was used to clean the mill between samples. Plant samples were then pre-weighed and prepared for ^{15}N and total nitrogen analysis. Analysis of all ^{15}N samples was conducted similar to the outlined methods in Mulvaney (1993) and Shearer and Kohl (1993). Natural abundance ^{15}N (O treatment), enriched ^{15}N abundance (N, A, *NA, N*A treatments), and total nitrogen were analyzed with elemental analyzers connected to continuous flow Stable Isotope Ratio Mass Spectrometers calibrated with IAEA reference standards. The mass spectrometer consisted of an automatic analyzer (Dumas flash combustion) for total nitrogen, and a flow-through system for nitrogen gas generated for the isotope ratio analysis, using a universal collector system. Output values were provided as atom% ^{15}N for enriched ^{15}N samples, and as $\delta^{15}N$ vs. air for natural abundance ^{15}N samples. Natural abundance values were converted to atom% ^{15}N using the relationship between atom% ^{15}N and $\delta^{15}N$:

$$\delta^{15}N = [\ (\text{atom\% }^{15}N/\text{atom \%}^{14}N)_{samp} \ /\ (\text{atom\% }^{15}N/\text{atom \%}^{14}N)_{std}\ ] \ -\ 1 * 1000$$

where (atom% ^{15}N/atom %^{14}N)$_{samp}$ was the isotope ratio of the sample to be determined and (atom% ^{15}N/atom %^{14}N)$_{std}$ was the isotope ratio of the standard, which is equivalent to N_2 in air (0.3666, International Atomic Energy Agency) (Hauck et al. 1994).

3.2.6.7. Mortality

After all seedlings had been used for necessary measurements, % mortality was calculated based upon the number of seedlings that died during the course of the experiment compared with the total number of seedlings that had been harvested and that were still living at the end of the experiments.

3.2.7. H. crustuliniforme enzyme assays

All chemicals for enzyme assays were purchased from Sigma-Aldrich (Canada). Enzyme assays were conducted with mycelial tissue grown on plates of N, A, NA and O treatment media. NR assays were conducted according to the methods of Wagner et al. (1989), with 0.1 g mycelia and an enzyme incubation time of two hours in the water bath. GS assays were conducted according to the methods of Truax et al. (1994) with 0.5 g mycelial tissue. Standard curves for NR and GS were prepared as for aspen samples, using known concentrations of KNO_2 (A_{540} nm) and L-glutamine-γ-hydroxamate (A_{540} nm), respectively.

NAD- and NADP-glutamate dehydrogenase (GDH) assays were conducted with 0.25 g mycelial tissue according to the methods outlined by Rudawska et al. (1994). Assays for NAD-GDH and NADP-GDH were performed using the same mycelial extract for each sample but with different assay buffers (Rudawska et al. 1994) that contained the same molar concentrations of either NADH or NADPH as an enzyme substrate (A_{340} nm).

Mycelial enzyme extracts were quantified for protein using Potty's modification of the Lowry protocol (Potty 1969) using bovine serum albumin as a standard (A_{500} nm). NR activity was calculated as nmol NO_2 produced h^{-1} mg^{-1} fungal protein. GS activity was calculated as mmol monohydroxamate produced h^{-1} mg^{-1} fungal protein. GDH activity was calculated as nkat mg^{-1} fungal protein (nkat = nmoles NAD(P)H consumed or produced s^{-1}).

3.2.8. Statistical analysis

All data were analyzed with SAS 9.1. (SAS Institute, North Carolina, USA) to determine statistically significant ($p \leq 0.05$) differences between treatment combinations of nitrogen form, level, and mycorrhizal inoculation for all experiments involving aspen seedlings, and between different nitrogen forms for the enzyme assay experiment with mycelial tissue. All data were first tested for normality of distribution and homogeneity of variance. Residuals from the data sets were calculated and used to graph normal probability plots and box plots, to determine if there were any statistical outlier values in the data sets. Outliers, if any, were removed from the data sets.

3.2.8.1. Aspen data

All data from aspen seedlings (described in sections 3.2.6.1. – 3.2.6.4.), with the exception of the enzyme assay and ^{15}N data, were analyzed for statistically-significant differences. To compare the effect of nitrogen to the effect of no nitrogen, and the effect of nitrogen*mycorrhiza to the effect of no nitrogen*mycorrhiza, each treatment combination (nitrogen form*level*mycorrhiza) was first considered as a separate treatment. Then each treatment was statistically compared to CTRL O and to MYCO O using Dunnett's test. To compare the effects of different nitrogen form*level*mycorrhiza treatment combinations with each other, treatments were analyzed using a MIXED ANOVA model of a 2 (mycorrhizae) x 2 (nitrogen level) x 4 (nitrogen form) factorial experiment within a randomized complete block (RCB) design (excluding CTRL O and MYCO O). A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment combination group harvested per day. Therefore, the days of measurements were the blocks.

The statistical model used for the RCB three-factor design was:

$$Y_{ijklm} = u + N_i + L_j + M_k + N_iL_j + N_iM_k + L_jM_k + N_iL_jM_k + B_l + e_{ijklm}$$

where:

Y_{ijklm} = value of individual observation (*i*=nitrogen, *j*=level, *k*=mycorrhizae, *l*=block, *m*=observation)

u = overall mean of observations

N_i = effect of i^{th} treatment (i = form of nitrogen)

L_j = effect of j^{th} treatment (j = level of nitrogen)
M_k = effect of k^{th} treatment (k = mycorrhizal or non-mycorrhizal treatment)
N_iL_j = interaction effect between i^{th} and j^{th} treatments
N_iM_k = interaction effect between i^{th} and k^{th} treatments
L_jM_k = interaction effect between j^{th} and k^{th} treatments
$N_iL_jM_k$ = interaction effect between i^{th}, j^{th}, and k^{th} treatments
B_l = effect of l^{th} block (l=day of measurement)
e_{ijklm} = residual error

Data from the NR and GS enzyme assays (described in section 3.2.6.5.) were analyzed using a mixed ANOVA model 2 (mycorrhizae) x 4 (nitrogen form) factorial experiment incorporated into a split-plot design (including CTRL O and MYCO O). Data were analyzed to compare significance between least-squares means of individual treatment combinations, and to compare between leaf and root samples within individual plants. A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment combination harvested per day. Therefore, the days of measurements were the blocks.

The statistical model used for the two-factor split-plot design was:

$$Y_{ijklm} = u + M_i + N_j + P_k + B_l + M_iN_j + M_iP_k + N_jP_k + M_iN_jP_k + Q_{il} + e_{ijklm}$$

where:

Y_{ijklm} = value of individual observation (i=mycorrhizae, j=nitrogen, k=part, l=block, m=observation)
u = overall mean of observations
M_i = main effect of i^{th} treatment (i = mycorrhizal or non-mycorrhizal treatment)
N_j = effect of j^{th} treatment (j = form of nitrogen)
P_k = sub-plot effect of k^{th} treatment (k = part of plant, either leaf or root)
B_l = effect of l^{th} block (l=day of measurement)
M_iN_j = interaction effect between i^{th} mycorrhizal treatment, j^{th} nitrogen form
M_iP_k = interaction effect between i^{th} mycorrhizal treatment, k^{th} plant part
N_jP_k = interaction effect between j^{th} nitrogen form, k^{th} plant part

$M_iN_kP_l$ = interaction effect between i^{th}, k^{th}, and l^{th} treatments

Q_{ij} = main plot error (interaction between i^{th} treatment and l^{th} block) = error a

e_{ijklm} = residual error associated with sub-plots within subplots = error b

Data from the GDH enzyme assays (described in section 3.2.6.5.) were analyzed for significant differences between treatment combinations using a mixed ANOVA model 2 (mycorrhizae) x 4 (nitrogen form) factorial RCB design (including CTRL O and MYCO O). A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment combination group harvested per day. Therefore, the days of measurements were the blocks.

The statistical model used for a two-factor RCB design was:

$Y_{ijkl} = u + M_i + N_j + M_iN_j + B_k + e_{ijkl}$ where:

Y_{ijkl} = value of individual observation (*i*=mycorrhizae, *j*=nitrogen, *k*=block, *l*=observation)

u = overall mean of observations

N_i = effect of i^{th} treatment (i = form of nitrogen)

M_j = effect of j^{th} treatment (j = mycorrhizal or non-mycorrhizal)

M_iN_j = interaction effect between i^{th} and j^{th} treatments

B_k = effect of k^{th} block (k=day of measurement)

e_{ijkl} = residual error

Enriched ^{15}N treatment data (described in section 3.2.6.6.) were analyzed for significant differences in ^{15}N levels between treatment combinations between leaves and roots of the same plant unit with a MIXED ANOVA model 2 (mycorrhizae) x 4 (nitrogen form) x 2 (plant part) factorial experiment incorporated into a complete randomized (CR) design (including CTRL O and MYCO O). The statistical model used for this analysis was:

$Y_{ijkl} = u + M_i + N_j + P_k + M_iN_j + M_iP_k + N_jP_k + M_iN_jP_k + e_{ijkl}$

where:

Y_{ijkl} = value of individual observation (i=mycorrhizae, j=nitrogen, k=plant part, l=observation)

u = overall mean of observations

M_i = main effect of i^{th} treatment (i = mycorrhizal or non-mycorrhizal treatment)

N_j = effect of j^{th} treatment (j = form of nitrogen)

P_k = effect of k^{th} treatment (k = part of plant, either leaf or root)

M_iN_j = interaction effect between i^{th} mycorrhizal treatment, j^{th} nitrogen form

M_iP_k = interaction effect between i^{th} mycorrhizal treatment, k^{th} plant part

N_jP_k = interaction effect between j^{th} nitrogen treatment, k^{th} plant part

$M_iN_jP_k$ = interaction effect between i^{th}, j^{th}, and k^{th} treatments

e_{ijkl} = residual error

Data from enriched ^{15}N seedling treatments (described in section 3.2.6.6.) were statistically compared to natural abundance ^{15}N treatment data and analyzed for significant differences between treatment combinations with a MIXED ANOVA model 2 (mycorrhizae) x 4 (nitrogen form) x 2 (enrichment) factorial experiment incorporated into a split-plot design (including CTRL O and MYCO O). The statistical model used for this analysis was:

$$Y_{ijklm} = u + M_i + N_j + P_k + E_l + M_iN_j + M_iP_k + N_jP_k + M_iN_jP_k + Q_{il} + e_{ijklm}$$

where:

Y_{ijklm} = value of individual observation (i=mycorrhizae, j=nitrogen, k=part, l=block, m=observation)

u = overall mean of observations

M_i = main effect of i^{th} treatment (i = mycorrhizal or non-mycorrhizal treatment)

N_j = effect of j^{th} treatment (j = form of nitrogen)

P_k = sub-plot effect of k^{th} treatment (k = part of plant, either leaf or root)

E_l = effect of l^{th} block (l=^{15}N enriched or not)

M_iN_j = interaction effect between i^{th} mycorrhizal treatment, j^{th} nitrogen form

M_iP_k = interaction effect between i^{th} mycorrhizal treatment, k^{th} plant part

N_jP_k = interaction effect between j^{th} nitrogen form, k^{th} plant part

$M_iN_kP_l$ = interaction effect between i^{th}, k^{th}, and l^{th} treatments

Q_{ij} = main plot error (interaction between i^{th} treatment and l^{th} block) = error a

e_{ijklm} = residual error associated with sub-plots within subplots = error b

Comparisons from the Dunnett's test (α=0.05) and MIXED ANOVA tests were conducted using least-squares means. For MIXED ANOVA, corresponding Tukey-adjusted p-values were used to further examine differences among the treatment combinations (nitrogen form*level*mycorrhiza) using pre-planned comparisons (α=0.05). The slice function was used to analyze and interpret interactions between main effects that were statistically significant (α=0.05) for multi-factor MIXED ANOVA. The results of all statistical analyses are presented in figures and tables at the end of this chapter.

3.2.8.2. H. crustuliniforme data

To statistically compare the effect of the different forms of nitrogen for each assay, data from NR, GS, and GDH assays (described in section 3.2.7.) were analyzed to compare the differences between the nitrogen treatments using a one-factor CR ANOVA. The statistical model used for this analysis was:

$Y_{ij} = u + N_i + e_{ij},$

where:

Y_{ij} = value of individual observation (*i*=nitrogen, *j*=observation)

u = overall mean of observations

N_i = effect of i^{th} treatment (*i* = form of nitrogen)

e_{ij} = residual error

Comparisons for the single-factor ANOVA were conducted using least-squares means (α=0.05). The results of statistical analyses are presented in figures and tables at the end of this chapter.

3.3. RESULTS

3.3.1. Shoot height, stem diameter, and leaf area

Least-squares means for shoot height for all mycorrhiza*nitrogen treatment combinations are shown (Fig. 3.1A). Shoot height significantly ($p \leq 0.05$) increased from low (2 mM) to high (8 mM) nitrogen concentration in both CTRL and MYCO seedlings for all nitrogen treatments, however the CTRL and MYCO N_8 and A_8 treatments had significantly greater mean shoot height than the 8 mM nitrate+ammonium (NA_8) treatment. There were no significant differences between CTRL and MYCO seedlings for the same nitrogen treatments, which was also indicated by the non-significant p-values for the main and interaction effects of mycorrhizae, although the other main effects (nitrogen, level) and interaction effect of nitrogen*level were highly ($p<0.0001$) significant (Tab. 3.1).

Stem diameter and leaf area also increased by ~ 30% with high (8 mM) concentrations of all treatments in both CTRL and MYCO seedlings, although only for N and A treatments were the increases significantly higher than O (Tab. 3.2). There were no significant differences between CTRL and MYCO seedlings for any of the nitrogen treatments. For stem diameter, only the main effect of nitrogen level was significant, whereas the main effects (nitrogen, level) and interaction effect (nitrogen*level) were significant for leaf area (Tab. 3.1).

3.3.2. Total chlorophyll and chlorophyll b:a ratio

Least-squares means for total chlorophyll concentrations for all treatment combinations are shown (Fig. 3.1B). Chlorophyll concentrations in leaves of CTRL and MYCO seedlings increased with increasing nitrogen concentrations for all nitrogen treatments relative to CTRL O (0.781 ± 0.0840 mg g^{-1} FW) and MYCO O (0.875 ± 0.120 mg g^{-1} FW). Although there was a slight increase in the 2 mM nitrogen MYCO and CTRL treatments relative to O, the 8 mM nitrogen treatments were significantly higher than O. Only in CTRL seedlings were there significantly higher means in the 8 mM nitrogen treatments relative to the 2 mM means. This is supported by the statistical significance of the main effect of level ($p<0.0001$) and the significant ($p<0.05$)

interaction effects (mycorrhiza*level, mycorrhiza*nitrogen*level) (Tab. 3.1). There were no significant differences between CTRL and MYCO seedlings for any of the nitrogen treatments.

Chlorophyll b:a ratios decreased with an increase in nitrogen concentrations for all nitrogen treatments relative to CTRL O (1.14 ± 0.161) and MYCO O (0.971 ± 0.167), with least-squares means shown (Fig. 3.1C). As with total chlorophyll, there were significant differences between 2 mM and 8 mM nitrogen treatments in CTRL seedlings, but not in MYCO seedlings, where the only significant differences were between the 8 mM means and O. There were no significant differences between CTRL and MYCO seedlings for any of the nitrogen treatments. Although the main effects (nitrogen, level, mycorrhiza) were significant, there were no significant interaction effects for chl b:a ratios (Tab. 3.3).

3.3.3. Root colonization, root volume, and root:leaf dry weight ratio

Percent root length colonization by *H. crustuliniforme* increased in nitrogen treatments in both CTRL and MYCO seedlings compared with CTRL O and MYCO O seedlings (Tab. 3.2). Differences between nitrogen treatments for either CTRL or MYCO seedlings were not significant, which was also indicated by the statistical significance of only one main effect (mycorrhiza, Tab. 3.3). Only for the N_8 treatment were MYCO seedling roots significantly more colonized than CTRL seedlings.

Mean root volume significantly increased in all the 8 mM nitrogen treatments in CTRL seedlings, but only in the A_8 treatment for MYCO seedlings, relative to CTRL O (2.06 ± 0.117 cm^3) and MYCO O (2.6 ± 0.368 cm^3) (Fig. 3.2A). Root volumes were similar between CTRL and MYCO seedlings within nitrogen treatments, with the exception of the N_8 treatment, where root volume was significantly higher for CTRL than for MYCO seedlings. All main effects showed statistical significance, as did some of the interaction effects (nitrogen*mycorrhiza, mycorrhiza*nitrogen*level) (Tab. 3.1).

Mean root:leaf dry weight ratios (Fig. 3.2B) significantly decreased for all 8 mM nitrogen treatments in both CTRL and MYCO seedlings relative to CTRL O (4.93 ± 1.18) and MYCO O (4.42 ± 0.542), but the 2 mM nitrogen treatments remained relatively unchanged compared with O means. There were no significant interaction effects, with only the main effect of level being significant (Tab. 3.3). There were no significant differences between CTRL and MYCO seedlings for the same nitrogen treatments.

3.3.4. Mortality

The nitrogen treatments with no mortality (0%) were NA_8 for both CTRL and MYCO seedlings, and N_8 and NA_2 for CTRL seedlings (Fig. 3.2C). For the other nitrogen treatments, with the exception of the A_8 treatment, percent mortality values were higher for all nitrogen MYCO treatments than for CTRL treatments. The treatments with highest percent mortality were 25.0 % (MYCO NA_2, A_2) and 15.0% (CTRL A_8).

3.3.5. pH of xylem exudate and sand leachate

Although the mean xylem exudate pH values of the 8 mM nitrogen treatments were slightly higher than those of the 2 mM nitrogen treatments in both CTRL and MYCO seedlings, the means did not differ significantly from O (5.95 ± 0.0910) in CTRL seedlings (Fig. 3.3A). In MYCO seedlings, only the xylem exudate pH for the A_8 treatment was significantly higher than for O. The pH range of xylem exudate was small, from 5.89 ± 0.207 (CTRL A_2) to 6.27 ± 0.0858 (CTRL NA_8).

Sand leachate pH means (Fig. 3.3B) for the 8 mM treatments tended to be slightly lower than those for 2 mM treatments, with significant differences between N_2 and N_8 in both CTRL and MYCO seedlings, and between A_2 and A_8 in MYCO seedlings. Sand leachate pH means for the A_8 treatment of both CTRL and MYCO seedlings were significantly lower compared with CTRL O (8.18 ± 0.277) and MYCO O (8.04 ± 0.280). The range of pH means from sand leachate was greater than for xylem exudate, ranging from 6.68 ± 0.0724 (MYCO A_8) to 8.43 ± 0.158 (CTRL N_2).

For pH means of both xylem exudate and for sand leachate, there were no significant differences between CTRL and MYCO seedlings within the same nitrogen treatment, and there were no significant interaction effects (Tab. 3.3).

3.3.6. *Nitrate and ammonium concentrations in xylem exudate*

While there were slight differences in nitrate and ammonium concentrations in xylem exudate between the different nitrogen treatments, most of these differences were not significant from CTRL O (0.0387 ± 0.0254 mg kg^{-1} NO_3^-, 0.708 ± 0.269 mg kg^{-1} NH_4^+) and MYCO O (0.185 ± 0.0828 mg kg^{-1} NO_3^-, 1.59 ± 0.142 mg kg^{-1} NH_4^+) seedlings (Fig. 3.4A,B). However, the mean NH_4^+ concentration of the CTRL A_8 treatment was significantly higher than that of the CTRL O and the MYCO A_8 treatments, and the MYCO N_8 mean NO_3^- concentration was significantly higher than that of the MYCO O and the CTRL N_8 treatments. Interaction effects were significant for NO_3^- (nitrogen*level, nitrogen*mycorrhiza, mycorrhiza*nitrogen*level) and for NH_4^+ (nitrogen*level, nitrogen*mycorrhiza, mycorrhiza*level) concentrations (Tab. 3.1). Although the slice function (slice=nitrogen) showed that these interactions differed by N for the NO_3^- concentrations, and by A for the NH_4^+ concentrations, as expected, the mycorrhiza*level interaction for NH_4^+ concentrations differed within CTRL seedlings but not MYCO seedlings (Tab. 3.1).

3.3.7. *Total nitrogen and ^{15}N measurements*

Overall, total nitrogen in leaves was greater than total nitrogen in roots (Fig. 3.5A), with significant differences between leaf and root for the N treatment in both CTRL and MYCO seedlings. Means were also significantly higher in leaves of N and A treatments of both CTRL and MYCO seedlings relative to CTRL O (0.870 ± 0.0165% DW) and MYCO O (0.802 ± 0.0275% DW). For roots, nitrogen means were significantly higher for the A treatment in CTRL seedlings and for the N, A, and N*A (N+^{15}A) treatments in MYCO seedlings relative to CTRL O (0.771 ± 0.0638 % DW) and MYCO O (0.619 ± 0.0387 % DW). There were no significant differences between leaves or roots of CTRL and MYCO seedlings for the same nitrogen treatment, and no significant interaction effects (Tab. 3.4).

Within CTRL and MYCO seedlings, leaves of N, A, and *NA had significantly higher enriched ^{15}N concentrations than the natural abundance ^{15}N concentrations in CTRL O (0.366 ± 0.000132 Atom % ^{15}N) and MYCO O (0.366 ± 0.000769 Atom % ^{15}N)

treatments (Fig. 3.5B). For CTRL seedlings, all nitrogen treatments had significantly higher root ^{15}N concentrations than for CTRL O (0.369 ± 0.00165 Atom % ^{15}N) seedling roots. Within MYCO seedlings, all nitrogen treatments except for the *NA treatment had higher root ^{15}N concentrations relative to MYCO O roots (0.369 ± 0.000771 Atom % ^{15}N). Although roots generally had higher mean ^{15}N concentrations than leaves for CTRL and MYCO seedlings, with the differences being significant for N and A treatments of CTRL seedlings, the opposite was true for N and A treatments in MYCO seedlings. There were no significant differences between leaf and root means for any nitrogen treatment in MYCO seedlings. Overall, mean ^{15}N concentrations were higher for MYCO than those for CTRL seedlings, with significant differences between N leaves, A leaves, and N roots. There were significant interaction effects (mycorrhiza*nitrogen, mycorrhiza*part) which differed for both CTRL and MYCO seedlings separately, and significant interaction effects (mycorrhiza*nitrogen*part) which differed by CTRL ammonium-treated seedlings and CTRL nitrate-treated seedlings (Tab. 3.4).

Natural abundance ^{15}N concentrations for roots and leaves of all of the nitrogen treatments for both CTRL and MYCO seedlings were also measured to ensure that base ^{15}N concentrations were similar between treatments prior to ^{15}N enrichment. There were no significant differences in natural abundance atom %^{15}N concentrations between CTRL and MYCO seedlings, between leaves and roots, between nitrogen treatments, or between any of the nitrogen treatments and O (data not shown). Natural abundance ^{15}N in treatments ranged between 0.392 ± 0.00074 Atom % ^{15}N (MYCO N) to 0.366 ± 0.00013 Atom % ^{15}N (CTRL O).

3.3.8. Enzyme assays

NR activity did not significantly change with nitrogen treatment in either leaves or roots of MYCO seedlings or in leaves of CTRL seedlings (Fig. 3.6A). However, in roots of CTRL seedlings, NR activity significantly increased ten-fold in N and fifteen-fold in NA treatments relative to O (24.2 ± 8.2 nmol NO_2 h^{-1} mg^{-1} protein). For the NA treatment, mean NR activity was significantly fifteen-fold higher in roots compared to the leaves within CTRL and MYCO seedlings, and mean NR activity was significantly 2-

fold higher in roots of CTRL than in MYCO (178.4 + 68.5 nmol NO_2 h^{-1} mg^{-1} protein) seedlings. There were no statistically-significant interaction effects, although one of the main effects (part) was significant (Tab. 3.4).

Mean GS activity did not change significantly with nitrogen treatment in leaves or roots of CTRL seedlings or in leaves of MYCO seedlings (Fig. 3.6B). In MYCO seedling roots, GS activity was significantly lower in A compared with NA treatments, although neither was significantly different from O (0.3 ± 0.08 mmol hydroxamate h^{-1} mg^{-1} protein). Mean GS activity in MYCO NA seedling roots was significantly 30% higher than that of the leaves within the same treatment. The only significant difference between CTRL and MYCO seedlings was within the NA treatment, with MYCO roots having a 30% higher activity than CTRL roots (0.3 ± 0.05 mmol hydroxamate h^{-1} mg^{-1} protein).

Mean NAD- and NADP-GDH activity did not significantly change with nitrogen treatment in MYCO seedling roots, but in CTRL roots, both NAD- and NADP-GDH (13.5 ± 3.0 nkat mg^{-1} protein) activity for the NA treatment increased significantly relative to NAD-GDH O (29.9 ± 5.4 nkat mg^{-1} protein) and NADP-GDH O (5.7 ± 1.1 nkat mg^{-1} protein) (Fig. 3.6C). There were two significant differences between CTRL and MYCO seedlings, since NAD-GDH activity in NA CTRL roots was significantly 30% higher than in NA MYCO roots (36.7 ± 8.3 nkat mg^{-1} protein), and NADP-GDH activity for O MYCO roots was 2-fold significantly higher than activity of O CTRL roots (5.7 ± 1.1 nkat mg^{-1} protein).

3.3.9. H. crustuliniforme enzyme assays

Mean NR activity of *H. crustuliniforme* mycelia grown on nitrogen-treated agar media did not significantly change with different nitrogen treatments (Fig. 3.7A). Mean GS activity decreased significantly in N and A treatments relative to O (0.267 ± 0.0217 mmol hydroxamate h^{-1} mg^{-1} protein) (Fig. 3.7B). Mean NAD- and NADP-GDH activity increased significantly in A treatment relative to NAD-GDH O (5.31 ± 0.393 nkat mg^{-1} protein) and NADP-GDH O activity (2.81 ± 0.519 nkat mg^{-1} protein). Although NAD-

and NADP-GDH activity in NA treatment was significantly lower than that of A treatment, it was not significantly lower than that of O.

3.4. DISCUSSION

Both form and level of nitrogen affected aspen growth. Low (2 mM) concentrations of both nitrate and ammonium resulted in very slight or no increases in several measurement parameters (leaf area, root volume, total chlorophyll of aspen seedlings), compared with the no nitrogen (O) treatment. In contrast, high (8 mM) concentrations resulted in significant increases in several growth parameters (shoot height, stem diameter, leaf area, total chlorophyll, root volume), increased nitrogen assimilation (total nitrogen, ^{15}N), lowered root:leaf dry weight and chlorophyll b:a ratios, and lower mortality than for the 2 mM treatments. It was expected that higher concentrations of nitrogen would result in both increased root and shoot growth, leading to higher root volumes but lower root:leaf ratios, because nutrient-deficient plants tend to increase root growth but not shoot growth (Green et al. 1994). The results of the present study were consistent with previous studies reporting higher root:shoot ratios in nutrient-deficient trees (Green et al. 1994, Hou et al. 2004). Generally, there were no differences with respect to the form of nitrogen (N, A, NA) on root growth, with the exception of the N treatment in CTRL seedlings, which had a significantly higher root volume than the A and NA treatments. All total nitrogen values, with the exception of the O treatment, were within previously reported ranges of 1.1 – 2% in leaves (Henry 1973, Wallander 2002), and 0.9 – 1.3% in roots (Wallander 2002).

Chlorophyll measurements may have been an indication of adequate nitrogen supply in the 8 mM nitrogen treatments, and of nitrogen deficiency in the 2 mM treatments, as indicated by the increases in total chlorophyll concentrations and the decreases in chlorophyll b:a ratios in the 8 mM treatments, relative to the 2 mM treatments. Although no relationship between the chlorophyll b:a ratio and the form and level of supplied inorganic nitrogen has previously been reported, it is known that an increasing chlorophyll b:a ratio can occur as a result of drought, salinity, and nutrient deficiency or excess (Haisel et al. 2006, Ranjbarfordoei et al. 2006, Tzvetkova and Hadjiivanova 2006).

It was evident that trembling aspen seedlings grew equally well when treated with high concentrations (8 mM) of either N or A. Both treatments had approximately similar means for several parameters (shoot height, stem diameter, leaf area, root volume, chlorophyll content). N and A treatments also had similar leaf and root concentrations of ^{15}N, although the A treatment had a higher total nitrogen concentration in roots than the N treatment. These data indicate that aspen seedlings were able to utilize either nitrate or ammonium when they were supplied separately at the whole-plant level. These results are in contrast to previous research that suggested aspen was ammonium-sensitive because of aspen's preference for nitrate vs. ammonium, as well as excess accumulation of ammonium at the cellular level (Min et al. 1999, 2000, Kronzucker et al. 2003).

Although aspen was capable of assimilating both nitrate and ammonium at the cellular level, aspen's preference for nitrate could be observed from the mortality data and the enzyme assay data. Ammonium-treated aspen had higher mortality, particularly in the A_8 treatment. Aspen was more responsive to the presence of nitrate than to ammonium, indicated by the greater differences in NR activity, compared with differences in GS or GDH activity, among the different nitrogen treatments. Rapid induction of NR, and increased NR activity in aspen leaves and roots, in response to nitrate have been previously reported (Pearson et al. 1989, Min et al. 1998). The fact that NAD-GDH activity, which catalyzes ammonium assimilation, was higher than NADP-GDH activity, which catalyzes degradation of glutamate to ammonium, in all treatments indicates that net ammonium assimilation was occurring in roots. However, the presence of GDH activity and its response to form of nitrogen in non-mycorrhizal roots was surprising, given that GDH was reported to be undetectable in non-mycorrhizal roots (Namysl et al. 1989, Pierleoni et al. 2001). From the enzyme assays, it also seemed that ammonium was being assimilated relatively equally between leaves and roots, whereas slightly more nitrate was being assimilated in roots than in leaves. These results suggest that nitrate and ammonium are assimilated differently in aspen.

Although both nitrate and ammonium could be assimilated in leaves and roots, nitrate was preferentially assimilated in roots. This was consistent with the xylem exudate data, which showed that very little nitrate was translocated to shoots in nitrogen-containing treatments, whereas the xylem exudate ammonium concentration of the A_8 treatment was significantly higher than in the other treatments. These results are somewhat in contrast to previous studies that reported the occurrence of nitrogen assimilation, particularly ammonium, primarily in tree root systems (Geβler et al. 1998, Siebrecht and Tischner 1999). However, some tree species, including trembling aspen, have been reported to shift their primary location of nitrogen assimilation from roots to leaves at higher concentrations (Peuke et al. 1996, Kruse et al. 2003). It is possible that at high ammonium concentrations, ammonium assimilation had switched to the leaves in the present study, which would have explained the increased ammonium concentration in xylem exudate (A_8 treatment).

When nitrate and ammonium were supplied together (NA treatment), aspen responded with reduced growth (reduced shoot height, leaf area), and nitrogen uptake (reduced total nitrogen and ^{15}N concentrations in leaves and roots), compared with the N or A treatments. In spite of reduced growth, no mortality was observed in either the NA_2 or NA_8 treatments. These results were consistent with a previous study that reported poor growth in field-grown hybrid poplar when fertilized with NH_4NO_3 (DesRochers et al. 2006), but in contrast to other studies that reported improved growth with the use of both nitrate and ammonium fertilization according to specific nitrate-ammonium ratios (Stadler et al. 1993, Schortemeyer et al. 1997, Rothstein and Cregg 2005). It is possible that nitrate uptake could have been inhibited by negative feedback if nitrate or nitrate-derived amino acids accumulated in the roots rather than being transported to shoots to prevent such accumulation (Imsande and Touraine 1994, Gniazdowska et al. 1999). However, none of the measurements taken in the present study could explain the reduced growth of aspen in the NA treatment.

In contrast with the reduced growth observed in the NA treatment, nitrogen assimilation was higher in this treatment, compared with the N and A treatments. NR and GDH activities were enhanced in the presence of both nitrate and ammonium.

Additionally, the ^{15}N study indicated that when ammonium and nitrate were supplied together, ^{15}N-ammonium was preferentially taken up and assimilated by leaves and roots, compared with ^{15}N-nitrate. These results suggest that the effects of NH_4NO_3 nutrition in aspen, both at the whole-plant and cellular level, are not well understood. Further study is needed to determine the effects of NH_4NO_3 fertilization in *Populus* spp., and to clarify how and why elevated NR and GDH activity in NA-treated aspen roots did not translate into increased nitrogen assimilation or seedling growth.

The results of the present study indicated that pH changes, as a result of the forms of nitrogen supplied in the different treatments, were likely not a factor in the form of nitrogen preferred by aspen seedlings. It had been previously suggested that the form of nitrogen preferred by plants was determined by the availability of different nitrogen forms in soils of different pH (Rygiewicz et al. 1984a,b; Brix et al. 2002), or because the uptake of different nitrogen forms can alter intracellular pH (Wilkinson and Davies 2002), which is used in the mediation of chemical signaling pathways (Wilkinson 1999). In the present study, xylem exudate pH remained fairly constant between the different nitrogen treatments, consistent with pH values (~5.8) for xylem exudate previously observed in *Populus* (Siebrecht and Tischner 1999). These results were consistent with previous studies that reported minimal cytosolic pH changes as a result of nitrogen fertilization (Gerendás et al. 1990, Crawford and Glass 1998, Schubert and Yan 2007) and in trees where nitrogen assimilation occurred primarily in roots (Lips 1997).

Although nitrogen form slightly affected sand leachate pH, the changes were likely not sufficient to suggest that pH was a factor in aspen's preference for form of nitrogen. Sand leachate from the N and A treatments had a lower pH compared with the other treatments, and 8 mM treatments had lower leachate pH values than the 2 mM treatments. These results were consistent with previous findings (DesRochers et al. 2003). Although the NA treatment had a slightly higher leachate pH, compared with the N and A treatments, the difference was not likely sufficient to explain the large reduction in aspen growth observed in the NA treatment.

Hc did not have much effect on aspen seedling growth, as measurement parameters for MYCO seedlings across all nitrogen treatments did not significantly differ from those of CTRL seedlings. However, Hc affected aspen seedlings in the 8 mM treatments by increasing nitrogen uptake. Higher total nitrogen concentrations in MYCO seedling leaves compared with CTRL leaves, and significantly higher ^{15}N concentrations in MYCO leaves and roots compared with CTRL seedlings, were observed. These results are consistent with other studies that reported increased growth and nitrogen assimilation in trees inoculated with mycorrhizae (reviewed in Smith and Read 1997, Perez-Moreno and Read 2001). This has been particularly true for conifers (Andersson et al. 1996, Wallander 2002, Duñabeitia et al. 2004).

Hc in pure culture seemed to be able to assimilate both nitrate and ammonium well, based on the results of the enzyme assays, which were consistent with previous studies of Hc (Sarjala 1990, Scheromm et al. 1990b). Nitrogen-metabolizing enzymes present in Hc mycelia showed that Hc was responsive to the presence of nitrate, as indicated by increased NR activity, as well as the presence of ammonium, as indicated by increased GDH activity. GS activity was also affected by nitrogen treatment, with a slight decrease in the presence of either N or A relative to the O treatment. While it is possible that GS may also be of importance in certain species of mycorrhizal species of fungi (Namysl et al. 1989, Pierleoni et al. 2001), the fact that GDH activity showed more of an increase in the presence of ammonium suggests that GDH was more involved in ammonium assimilation in Hc mycelia. It is possible that the reduced GDH activity in the NA treatment was due to increased rates of nitrogen assimilation, which resulted in higher protein content of mycelial extracts and therefore lower GDH activity as expressed per mg of fungal protein. Extracts of NA-treated Hc that were used for enzyme assays had approximately twice the average protein concentration of mycelial extracts from the other nitrogen treatments (data not shown). As was the case in aspen roots, higher NAD-GDH activity compared with NADP-GDH activity indicates that net ammonium assimilation was occurring.

The results of the fungal enzyme assays are consistent with previous research, where fungal NR activity had increased in response to nitrate (Brunner et al. 2000), and was not suppressed by the presence of ammonium, nor required induction in basidiomycetes (Sarjala 1990, Scheromm et al. 1990b). GDH responded to N and A treatments as previously reported by Quoreshi et al. (1995). However, the observed decrease in GS activity in the N and A treatments was in contrast to previous findings (Quoreshi et al. 1995).

By examining and comparing the results of the Hc and aspen enzyme assays, it seemed that Hc enhanced nitrogen assimilation, particularly with respect to ammonium, but may have resulted in reduced nitrate assimilation in MYCO seedlings. NR activity in MYCO seedlings did not significantly differ between nitrogen treatments, although NR responded with increased activity in the presence of nitrate as expected. There was more NR activity in MYCO roots than in leaves, indicating that most of the nitrate assimilation was occurring in roots, consistent with the observations of Min et al. (1998). However, NR activity was lower in MYCO than in CTRL seedlings, particularly in the NA treatment, suggesting that less nitrate may have been assimilated in MYCO seedlings. These results are consistent with the xylem exudate data, which showed that nitrate translocation to shoots increased in the N_8 treatment in MYCO seedlings compared to CTRL seedlings. In the long-term, reduced nitrate assimilation may be a disadvantage for Hc-colonized aspen, as N_8-treated MYCO seedlings also had higher mortality compared to N_8-treated CTRL seedlings.

GS and GDH activities were also largely unaffected by the different nitrogen treatments in MYCO seedlings. As with CTRL seedlings, the results suggest that ammonium was being assimilated relatively equally in leaves and roots, and that net ammonium assimilation was occurring in MYCO roots (higher NAD-GDH activity). GS and GDH activities in MYCO seedlings were generally similar to those of CTRL seedlings for most nitrogen treatments, with a few exceptions. Hc resulted in increased GS activity in the NA treatment, increased GDH activity in the O treatment, and decreased GDH activity in the NA treatment. These results, along with the Hc enzyme assay results, indicate that Hc facilitated both nitrate and ammonium assimilation in aspen. Hc's responsiveness to ammonium, combined with aspen's responsiveness to nitrate, likely resulted in MYCO aspen being capable of enzymatically responding equally to the presence of both nitrate and ammonium. As a result, there were minimal differences in the relative activities of these three key enzymes involved in nitrogen metabolism (NR, GS, GDH) regardless of the nitrogen treatment. Increased ammonium assimilation in MYCO seedlings was also evident from the xylem exudate data, where MYCO seedlings in the A_8 treatment had low xylem ammonium concentrations compared with CTRL seedlings. Increased ammonium assimilation may be advantageous for Hc-colonized aspen in the long-term, as indicated by the reduced mortality in the A_8-treated MYCO seedlings compared to A_8-treated CTRL seedlings. These results of mycorrhizal effects upon plant ammonium assimilation are consistent with previous studies (Finlay et al. 1988, Chalot et al 1990b, Chalot et al. 1991).

It should also be noted that the enzyme assays conducted with MYCO seedlings did not take into account enzyme activity of any extraradical mycelia that may have been associated with roots. It is possible that enzyme activity may have been higher than that observed in Hc-inoculated seedlings, because previous studies have reported increased GDH activity in hyphal tissue connected to spruce roots than in the roots themselves (Brunner et al. 2000). Additionally, not much is known about the extent to which ECM fungi may assimilate nitrogen within hyphal tissue before transferring assimilates to their plant host roots, but this continues to be a controversial subject (Marmeisse et al. 2004, Montanini et al. 2006, Couturier et al. 2007). However, if mycorrhizal fungi are responsible for the majority of nitrogen assimilation in their fungal tissue, it would render mycorrhizal root enzyme activity less important for nitrogen assimilation, and could result in the down-regulation of the plant host's own nitrogen-metabolizing enzymes. This could also have resulted in the lack of differences in enzyme activities between the different nitrogen treatments in MYCO seedlings.

In spite of increased nitrogen uptake and assimilation in Hc-inoculated aspen, there appeared to be a negative effect of having a mycorrhizal association for MYCO seedlings in the O and 2 mM nitrogen treatments, as well as the N_8 treatment. MYCO seedlings in these treatments had higher mortality compared to CTRL seedlings in these treatments. Root volume for the N_8 treatment was also significantly lower for MYCO than for CTRL seedlings. These data, in the larger context of this study, indicate two possible drawbacks to aspen having an association with Hc: 1) although an association with Hc may lead to increased overall nitrogen assimilation under high nitrogen conditions, the fungus may be an additional stress for nutrient-starved seedlings because of its own separate nutrient requirements (discussed in Jones and Smith 2004); 2) although MYCO seedlings in the 8 mM treatments appeared to have equivalent growth and superior nitrogen assimilation compared to CTRL seedlings, seedling health was affected by the form of supplied nitrogen. When MYCO seedlings were given sufficient ammonium concentrations, they exhibited lower mortality than ammonium-treated CTRL seedlings. However, when treated with sufficient nitrate concentrations, MYCO seedlings had higher mortality than did CTRL seedlings.

The results of the present study indicate that it is unlikely Hc could affect the form of nitrogen preferred by aspen by producing pH changes within plants or within the soil. pH measurements of xylem exudate and sand leachate from MYCO seedlings were not significantly different from those of CTRL seedlings. Plant roots are capable of modifying rhizospheric pH (Zaccheo et al. 2006) in response to form of nitrogen present and taken up by roots (Marschner et al. 1991, DesRochers et al. 2003, Silber et al. 2004). Although there is evidence that some mycorrhizae can act as a rhizospheric pH buffer by altering the charge-balancing extrusion of OH^- ions or increasing the uptake of cations (Rygiewicz et al. 1984a,b), there was no evidence of Hc affecting soil pH in the present study. Therefore, any effects of Hc on aspen's response to form of nitrogen provided were not due to pH alteration.

Even though colonization rates between MYCO and CTRL seedlings were largely non-significant, MYCO seedlings had ~1.5-fold higher root colonization than did CTRL seedlings. The colonization of non-mycorrhizal control roots is indicative of the difficulties of maintaining a sterile environment for control seedlings, which has also been previously reported (Nardini et al. 2000). The lack of significant differences in the colonization rates may have been due to the non-specific staining technique used (Brundrett et al. 1996), which may have also resulted in the staining of fungi (mycorrhizal or saprophytic) other than Hc that may have been present in the pots and on roots at the time of harvest. Additionally, only a small number of seedlings were sub-sampled for root colonization measurements, therefore the small sample sizes may have resulted in the larger calculated standard errors for the MYCO treatments. Despite the small differences in root colonization percentages between CTRL and MYCO seedlings, Hc did have an effect on nitrogen uptake and assimilation of aspen seedlings.

3.4.1. Conclusions

The present study clearly showed that trembling aspen seedlings could assimilate and utilize both nitrate and ammonium to an almost equal extent in support of their growth, when either form of nitrogen was provided as the only nitrogen source at high (8 mM) concentrations. However, with respect to the enzymatic assimilation of nitrogen, aspen was more responsive to the presence of nitrate (NR activity) compared to ammonium (GS, GDH activity), indicating that aspen's preference for nitrate can be observed at the cellular level. In contrast, when nitrate and ammonium were provided together, overall seedling growth decreased while aspen's enzymatic assimilation of ammonium in these treatments increased. The nitrogen preference of aspen was not likely due to pH changes associated with the different forms of nitrogen.

Although Hc associated with aspen roots did not result in increased seedling growth in any of the nitrogen treatments, MYCO seedlings did show increased total nitrogen and ^{15}N uptake. Additionally, enzymatic nitrogen assimilation in MYCO seedlings was less affected by form of nitrogen compared to CTRL seedlings. This was likely due to Hc's ability to enzymatically respond to and assimilate ammonium which, when combined with aspen's enzymatic responsiveness to nitrate, was likely the reason that MYCO seedlings were capable of assimilating both nitrate and ammonium equally. The results of this study suggest that mycorrhizal benefits to aspen were mostly an increase in nitrogen uptake and assimilation, particularly with respect to ammonium, as well as a decrease in the percent mortality of ammonium-treated aspen. However, when nitrogen concentrations were low, ECM associations resulted in increased mortality of MYCO seedlings, and when only nitrate (N treatment) was present. It also appeared that MYCO seedlings were slightly less capable of assimilating nitrate than CTRL seedlings, as indicated by the shift in nitrate translocation to the shoot via xylem sap (MYCO) from ammonium translocation (CTRL).

The findings of this study suggest that aspen is not as ammonium-sensitive as previously considered by others (Min et al. 1999, 2000, Kronzucker et al. 2003), and that Hc may assist trembling aspen in the utilization of ammonium from boreal forest soils (Vitousek et al. 1982, Jerabkova et al. 2006). These findings also have important implications for aspen stand management with respect to nitrogen fertilization, given that ammonium nitrate resulted in reduced growth, as well as for the potential usefulness of ECM fungi in the nitrogen nutrition of managed stands. The cellular vs. whole-plant effects of nitrate and ammonium nutrition of aspen need to be studied further because of the discrepancy between cellular and whole-plant effects observed in the present study. The cellular and molecular ammonium transportation and assimilation mechanisms within the mycorrhizal-plant system, which are not currently understood (Shachar-Hill 2007), also merit further research.

3.5. LITERATURE CITED

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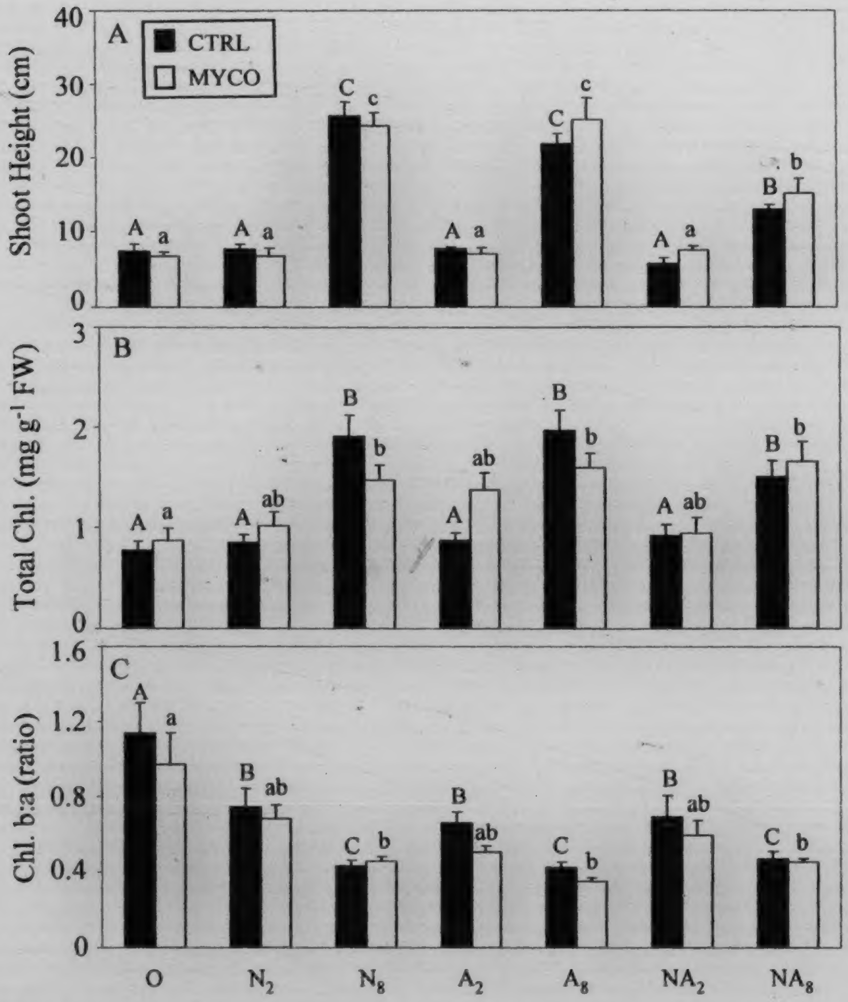

Figure 3.1. (**A**) Shoot height, (**B**) total chlorophyll, and (**C**) chlorophyll b:a ratio, in non-mycorrhizal (CTRL) and mycorrhizal (MYCO) aspen seedlings. Nitrogen treatments were either no nitrogen (O), or a combination of nitrogen form and concentration. Nitrogen forms were nitrate (N), ammonium (A), or nitrate + ammonium (NA). Nitrogen concentrations were either 2 mM ($_2$) or 8 mM ($_8$). Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments within CTRL seedlings), lowercase letters (among nitrogen treatments within MYCO seedlings), and asterisks (between CTRL and MYCO for each nitrogen treatment).

Table 3.1. ANOVA table indicating p-values of main effects and interactions of response variables to nitrogen form (nitrate, ammonium, nitrate+ammonium), nitrogen level (2 mM, 8 mM), and mycorrhiza (yes, no) treatment combinations in aspen seedlings. Effects were tested for significance at $\alpha=0.05$ using PROC MIXED factorial ANOVA (SAS 9.1). Only response variables with at least one significant ($p \leq 0.05$) effect are shown. Superscripts indicate significant ($p \leq 0.05$) slice operation results from SAS. For 'N form*N level' interaction, there were significant differences between levels within nitrate-only (^{N}), ammonium-only (^{A}), and nitrate+ammonium ($^{N+A}$) treatments. For 'N form*mycorrhiza' interaction, there were significant differences between mycorrhizal treatment within nitrate-only (^{N}), ammonium-only (^{A}), and nitrate+ammonium ($^{N+A}$) treatments. For 'mycorrhiza*N level' interaction, there were significant differences between nitrogen treatments within non-mycorrhizal (^{C}) and mycorrhizal (^{M}) treatments. For 'mycorrhiza*N form*N level' interaction, there were significant differences between levels within mycorrhizal+ammonium-only treatment combinations (^{M*A}) and within non-mycorrhizal+ammonium+nitrate treatment combinations ($^{C*N+A}$).

	Response Variables				
Effect	Leaf Area	Total Chlorophyll	Shoot Height	Stem Diameter	Root Volume
N Form	$p<0.01$	0.1463	$p<0.0001$	0.2018	$p<0.05$
N Level	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$
Mycorrhiza	0.6912	0.9862	0.4348	0.2125	$p<0.05$
N Form*N Level	$p<0.01^{N\ A\ N+A}$	0.8282	$p<0.0001^{N\ A\ N+A}$	0.4285	0.2313
N Form*Mycorrhiza	0.9885	0.5101	0.2864	0.3228	$p<0.05^{N\ A\ N+A}$
Mycorrhiza* N Level	0.9406	$p<0.01^{C\ M}$	0.4555	0.7806	0.8574
Mycorrhiza*N Form*N Level	0.9394	$p<0.05^{M*A}$	0.4973	0.945	$p<0.05^{C*N+A}$

Table 3.2. Stem diameter (Stem Dia.), leaf area (Leaf Area), and root colonization (Rt. Col.) in mycorrhizal (MYCO) and non-mycorrhizal (CTRL) aspen seedlings receiving either no nitrogen (O), or one of the following nitrogen treatments: 2 mM nitrate (N_2), 8 mM nitrate (N_8), 2 mM ammonium (A_2), 8 mM ammonium (A_8), 2 mM nitrate + ammonium (NA_2), or 8 mM nitrate + ammonium (NA_8). Least-squares means ± SE are shown (minimum n=8). Significant ($p \leq 0.05$) differences indicated by lowercase letters (between nitrogen treatments within either MYCO or CTRL seedling treatments separately) and asterisks (between MYCO and CTRL treatments for the same nitrogen treatment).

MYCO

Measurements	O	N_2	N_8	A_2	A_8	NA_2	NA_8
Stem Dia. (mm)	2.1±0.1a	2.0±0.3a	3.3±0.3b	2.1±0.2a	2.9±0.3ab	2.1±0.1a	3.0±0.3b
Leaf Area (cm^2)	21.9±2.3a	19.8±3.5a	151.6±20.2c	27.3±2.8ab	121.0±19.8c	20.5±2.5a	75.0±15.5b
Rt. Col. (%)	45.7±3.4a	44.4±0.7a	51.6±5.0a	36.4±11.0a	50.4±3.1a	46.8±16.4a	51.4±4.0a

CTRL

Measurements	O	N_2	N_8	A_2	A_8	NA_2	NA_8
Stem Dia. (mm)	2.2±0.2a	2.0±0.1a	3.2±0.3b	2.0±0.3a	3.0±0.2b	1.6±0.2a	2.6±0.3ab
Leaf Area (cm^2)	20.1±3.2a	28.9±2.9ab	151.4±24.1cd	27.2±1.38ab	124.2±23.4bc	23.0±3.7a	79.8±6.0ab
Rt. Col. (%)	22.6±0.4a	22.0±2.3a	28.0±1.1a*	42.4±7.5a	30.6±2.0a	32.3±2.4a	39.6±11.3a

Table 3.3. ANOVA table indicating p-values of main effects and interactions of response variables to nitrogen form (nitrate, ammonium, nitrate+ammonium), nitrogen level (2 mM, 8 mM), and mycorrhiza (yes, no) treatment combinations in aspen seedlings. Effects were tested for significance at $\alpha=0.05$ using PROC MIXED factorial ANOVA (SAS 9.1). Only response variables with at least one significant ($p\leq0.05$) effect are shown. Superscripts indicate significant ($p\leq0.05$) slice operation results from SAS. For 'N form*N level' interaction, there were significant differences between levels within nitrate-only (^{N}), and ammonium-only (^{A}) treatments. For 'N form*mycorrhiza' interaction, there were significant differences between mycorrhizal treatments within nitrate-only (^{N}) and within ammonium-only (^{A}) treatments. For 'mycorrhiza*N level' interaction, there were significant differences between nitrogen treatments within non-mycorrhizal (^{C}) treatments. For 'mycorrhiza*N form*N level' interaction, there were significant differences between levels within mycorrhizal+nitrate-only treatment combinations (^{M*N}).

	Response Variables						
Effect	Chl. b:a	Root Colonization	Root:Leaf DW	pH Leachate	pH Xylem Exudate	Xylem $[NO_3^-]$	Xylem $[NH_4^+]$
N Form	$p<0.05$	0.5482	0.3638	$p<0.0001$	0.9646	$p<0.05$	$p<0.05$
N Level	$p<0.0001$	0.3119	$p<0.0001$	$p<0.0001$	$p<0.05$	0.07252	$p<0.05$
Mycorrhiza	$p<0.05$	$p<0.01$	0.34405	0.5720	0.07062	0.1541	0.1660
N Form*N Level	0.46167	0.85241	0.4747	0.1942	0.9205	$p<0.05^{N}$	$p<0.05^{A}$
N Form*Mycorrhiza	0.4498	0.3467	0.9038	0.4728	0.1828	$p<0.05^{N}$	$p<0.05^{A}$
Mycorrhiza* N Level	0.1609	0.35602	0.6224	0.9660	0.8662	0.06008	$p<0.05^{C}$
Mycorrhiza*N Form*N Level	0.9999	0.3649	0.8342	0.05278	0.9452	$p<0.05^{M*N}$	0.06677

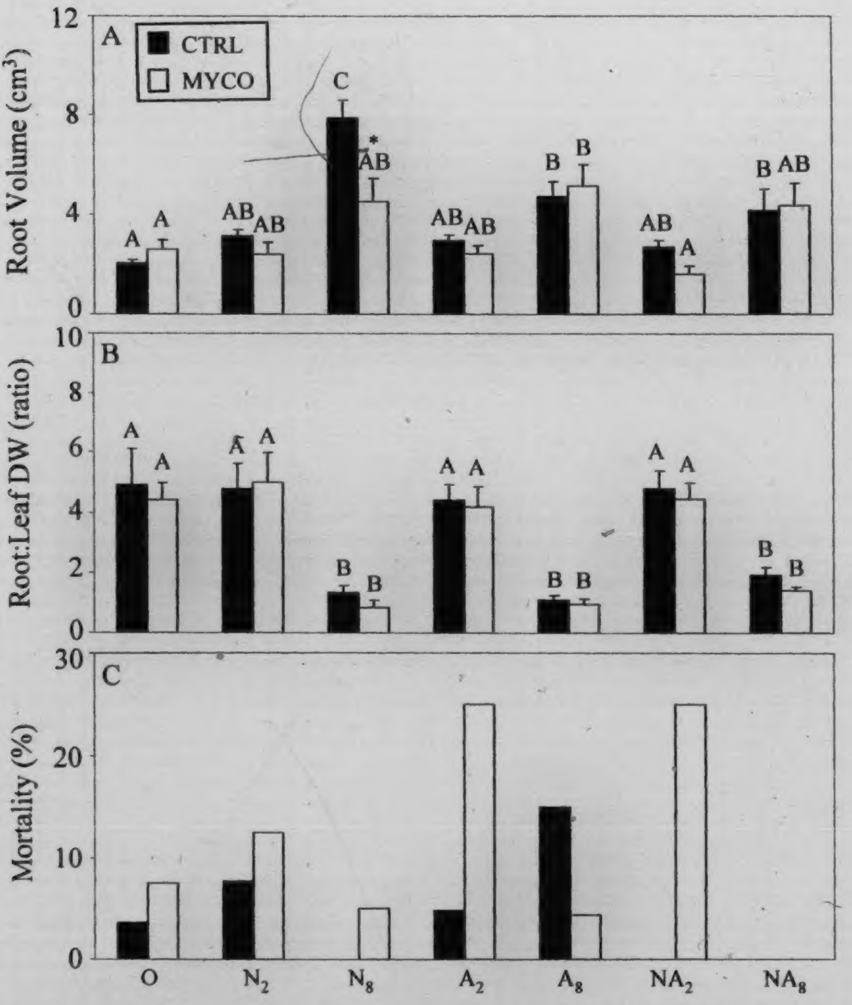

Figure 3.2. (**A**) Root volume, (**B**) root:leaf dry weight ratio, and (**C**) seedling mortality in non-mycorrhizal (CTRL) and mycorrhizal (MYCO) aspen seedlings. Nitrogen treatments were either no nitrogen (O), or a combination of nitrogen form and concentration. Nitrogen forms were nitrate (N), ammonium (A), or nitrate + ammonium (NA). Nitrogen concentrations were either 2 mM ($_2$) or 8 mM ($_8$). Least-squares means ± SE are shown, n=3 (**Fig. 3.2A**), minimum n=5 (**Fig. 3.2B, C**). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments within CTRL seedlings), lowercase letters (among nitrogen treatments within MYCO seedlings), and asterisks (between CTRL and MYCO for each nitrogen treatment).

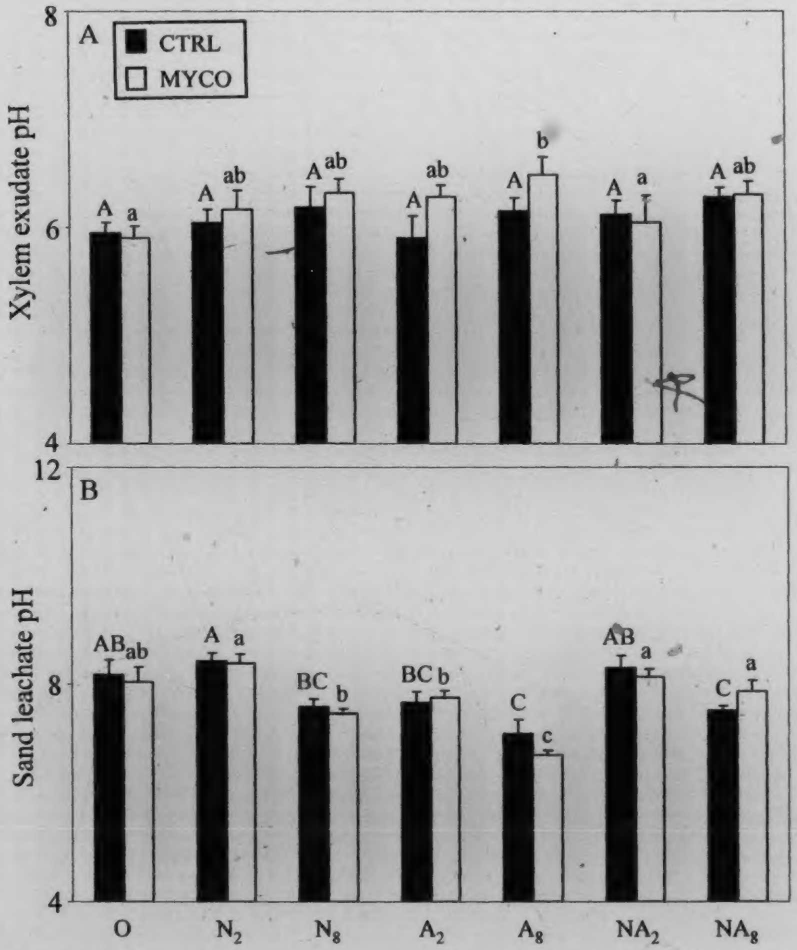

Figure 3.3. pH of xylem exudate (**A**) and sand leachate (**B**) in non-mycorrhizal (CTRL) and mycorrhizal (MYCO) aspen seedlings. Nitrogen treatments were either no nitrogen (O), or a combination of nitrogen form and concentration. Nitrogen forms were nitrate (N), ammonium (A), or nitrate + ammonium (NA). Nitrogen concentrations were either 2 mM ($_2$) or 8 mM ($_8$). Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments within CTRL seedlings), lowercase letters (among nitrogen treatments within MYCO seedlings), and asterisks (between CTRL and MYCO for each nitrogen treatment).

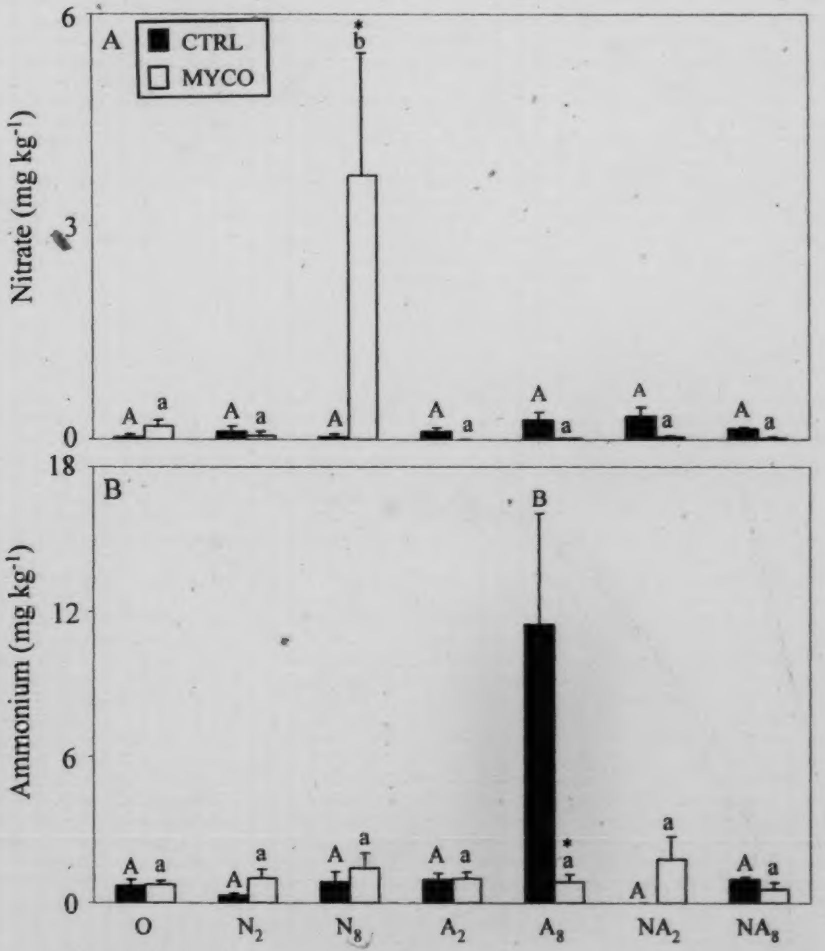

Figure 3.4. Xylem exudate concentrations of (**A**) nitrate and (**B**) ammonium in non-mycorrhizal (CTRL) and mycorrhizal (MYCO) aspen seedlings. Nitrogen treatments were either no nitrogen (O), or a combination of nitrogen form and concentration. Nitrogen forms were nitrate (N), ammonium (A), or nitrate + ammonium (NA). Nitrogen concentrations were either 2 mM ($_2$) or 8 mM ($_8$). Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments within CTRL seedlings), lowercase letters (among nitrogen treatments within MYCO seedlings), and asterisks (between CTRL and MYCO for each nitrogen treatment).

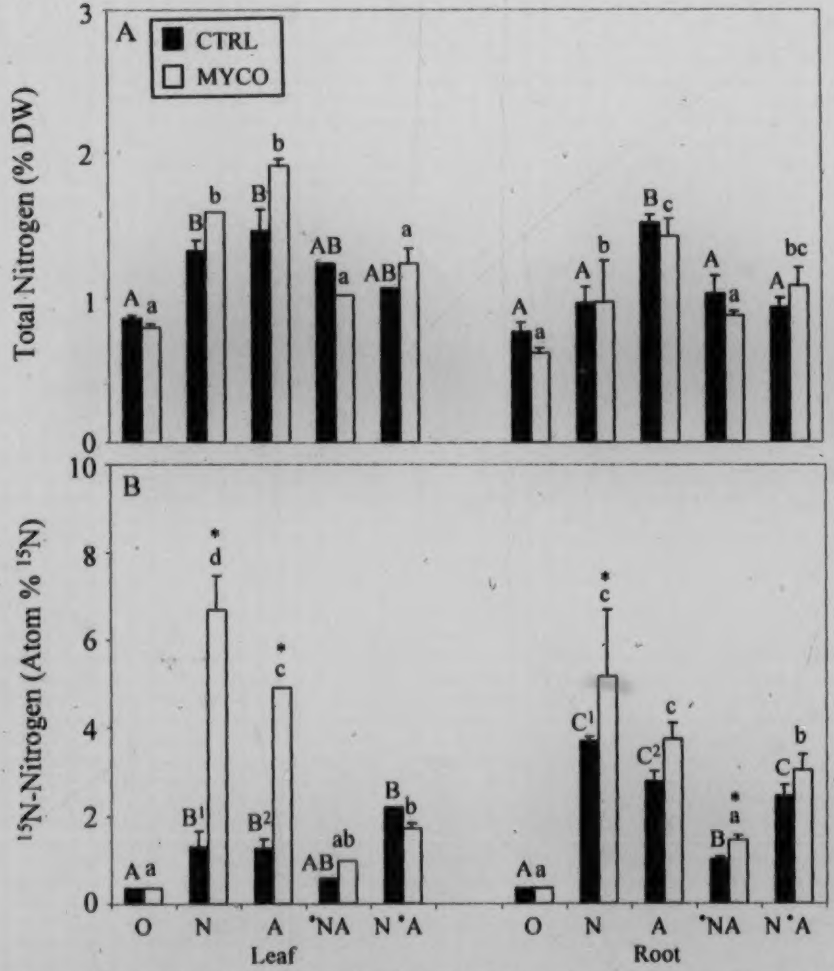

Figure 3.5. (**A**) Total nitrogen and (**B**) ^{15}N –nitrogen in dried leaf and root tissues of non-mycorrhizal (CTRL) and mycorrhizal (MYCO) aspen seedlings. Seedlings were treated with no nitrogen (O) or 8 mM of one of three forms of nitrogen: nitrate (N), ammonium (A), and nitrate + ammonium (NA). N, A, and NA seedling[illegible]ere given nutrient solutions containing $^{15}NO_3$ ($\dot{N}$), $^{15}NH_4$ ($\dot{A}$), and $NH_4{}^{15}NO_3$ (•NA) or $^{15}NH_4NO_3$ (N•A), respectively, for 72 hours prior to harvesting. Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments within CTRL seedlings), lowercase letters (among nitrogen treatments within MYCO seedlings), asterisks (between CTRL and MYCO for each nitrogen treatment), and subscript numbers (same number = significant difference between leaf and root for the same nitrogen*mycorrhiza treatment combination).

Table 3.4. ANOVA table indicating p-values of main effects and interactions of response variables to mycorrhiza (yes, no), nitrogen form (nitrate, ammonium, nitrate+ammonium), and part (leaf, root) treatment combinations in aspen seedlings. Effects were tested for significance at $\alpha=0.05$ using PROC MIXED factorial split-plot ANOVA (SAS 9.1). Only response variables with at least one significant ($p \leq 0.05$) effect are shown. Superscripts indicate significant ($p \leq 0.05$) slice operation results from SAS. For 'mycorrhiza*N form' interaction, there were significant differences between nitrogen form treatments within non-mycorrhizal (^{C}) and within mycorrhizal (^{M}) treatments. For 'mycorrhiza* part' interaction, there were significant differences between parts within non-mycorrhizal (^{C}) and within mycorrhizal (^{M}) treatments. For 'mycorrhiza*N form*part' interaction, there were significant differences between parts within non-mycorrhizal+ammonium-only treatment combinations (^{C*A}) and within non-mycorrhizal+nitrate-only treatment combinations (^{C*N}).

	Response Variables		
Effect	Nitrate Reductase Assay	Atom ^{15}N %	Total N %
Mycorrhiza	0.2185	$p<0.0001$	0.3583
N Form	0.07555	$p<0.0001$	$p<0.01$
Part	$p<0.01$	0.1336	$p<0.01$
Mycorrhiza*N Form	0.1410	$p<0.01^{CM}$	0.7399
Mycorrhiza*Part	0.5005	$p<0.05^{CM}$	0.2402
N Form*Part	0.07510	0.9258	0.3300
Mycorrhiza*N Form *Part	0.2864	$p<0.05^{C*A,C*N}$	0.5384

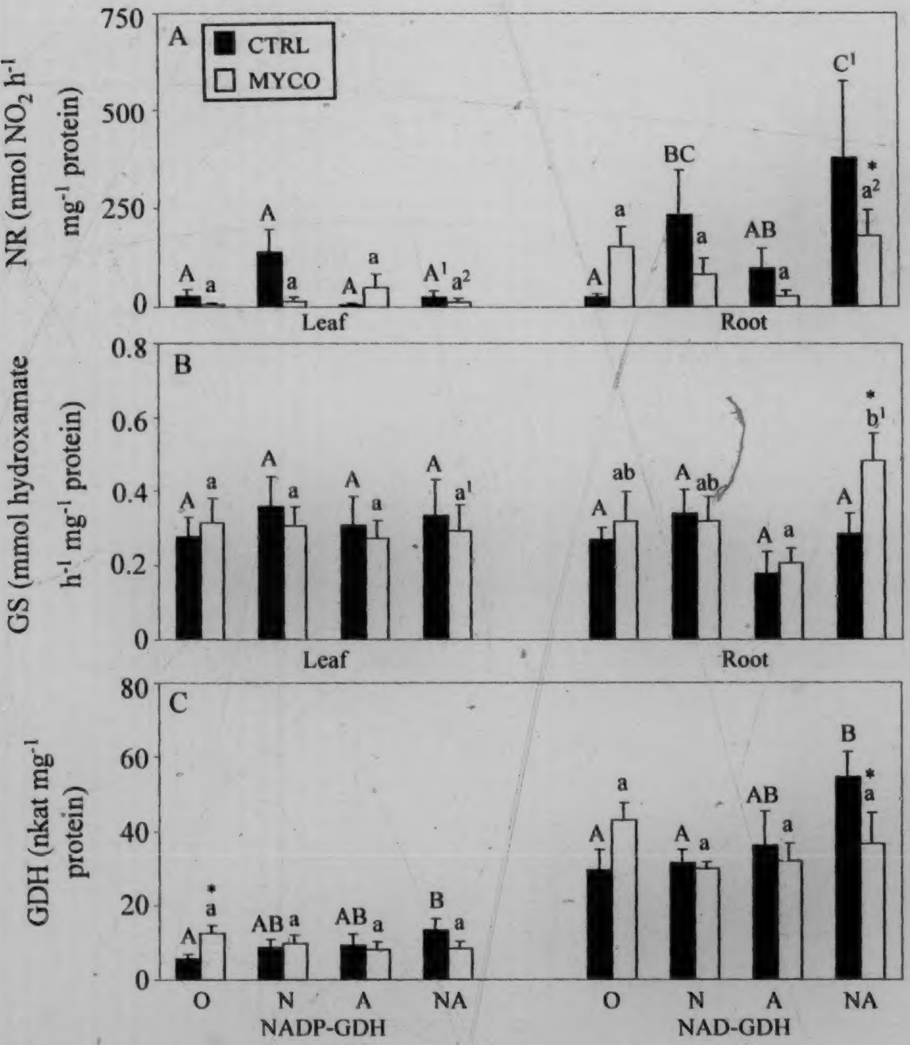

Figure 3.6. Leaf and root enzyme activity of (**A**) nitrate reductase, (**B**) glutamine synthetase, and (**C**) root NADP- and NAD-glutamate dehydrogenase activity in non-mycorrhizal (CTRL) and mycorrhizal (MYCO) aspen seedlings. Nitrogen treatments were no nitrogen (O), or 8 mM of nitrate (N), ammonium (A), or nitrate + ammonium (NA). Least-squares means ± SE are shown (minimum n=5). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among nitrogen treatments within CTRL seedlings), lowercase letters (among nitrogen treatments within MYCO seedlings), asterisks (between CTRL and MYCO for each nitrogen treatment), and subscript numbers (same number = significant difference between leaf and root for the same nitrogen*mycorrhiza treatment combination).

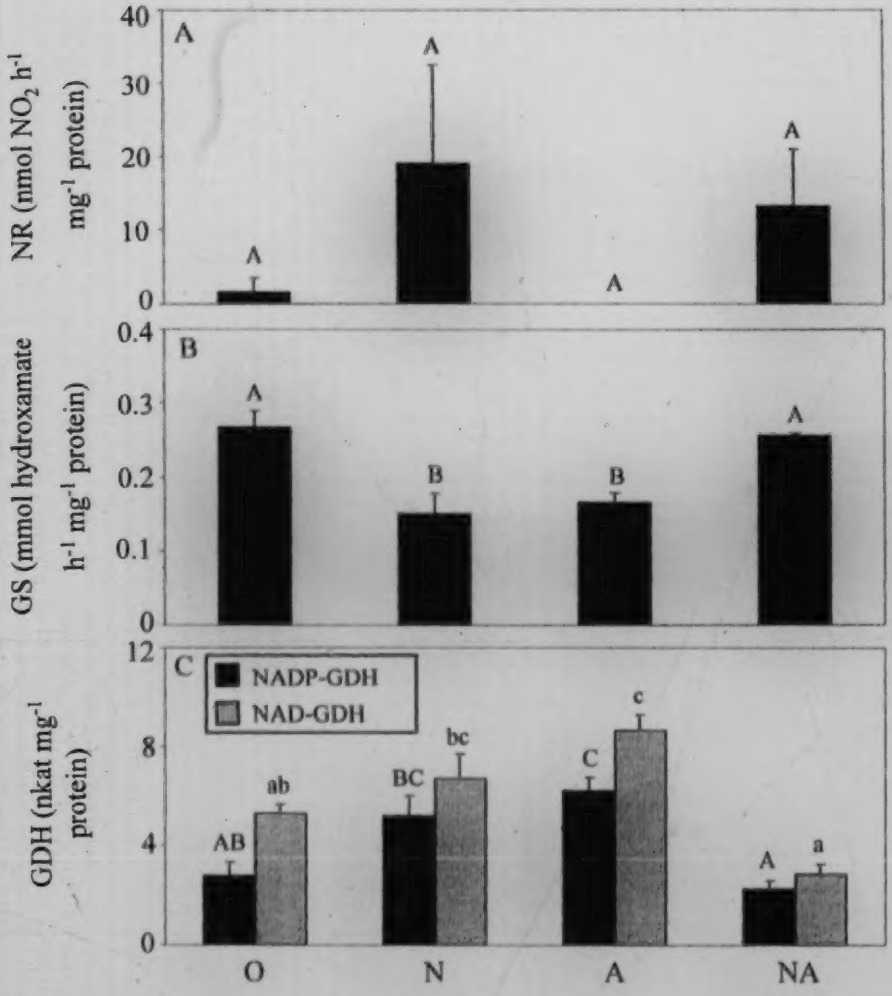

Figure 3.7. (**A**) nitrate reductase (NR), (**B**) glutamine synthetase (GS), and (**C**) NADP- and NAD-glutamate dehydrogenase (GDH) activity in *Hebeloma crustuliniforme* mycelia grown on nutrient agar containing either no nitrogen (O) or 8 mM of nitrate (N), ammonium (A), or nitrate + ammonium (NA). Least-squares means ± SE are shown (minimum n=4). Significant ($p \leq 0.05$) differences between nitrogen treatments indicated by uppercase letters for **Fig. 3.7A,B**. For **Fig. 3.7C**, significant differences between nitrogen treatments for NADP-GDH are indicated by uppercase letters, and significant differences between nitrogen treatments for NAD-GDH are indicated by lowercase letters.

CHAPTER IV

Relationship between pH tolerance of *Hebeloma crustuliniforme* and the effect of pH on root water flow properties in ectomycorrhizal trembling aspen (*Populus tremuloides)* seedlings

4.1. INTRODUCTION

Environmental pH changes due to anthropogenic factors, including acid rain (Kukkola et al. 2005, Sant'Anna-Santos et al. 2006), soil liming and other soil amendments (Huber et al. 2006, Rigueiro-Rodriguez et al. 2007), and mining operations (Schaaf and Huttl 2005, Kuznetsova and Mandre 2006) can have immediate and long-term effects on tree physiology and forest health (Skousen et al. 1994, Westbrook et al. 2006). Plant response to pH depends upon a plant's ability to control its apoplastic and intracellular pH, which is a mediator of chemical signaling pathways that in turn will determine the plant's response to environmental stresses (Wilkinson 1999). The forms of available nitrogen in forest soils can also influence apoplastic and rhizospheric pH through plant uptake (Wilkinson and Davies 2002, DesRochers et al. 2003, Silber et al. 2004). Although it is known that pH can indirectly affect tree physiology by influencing nutrient and heavy metal solubility (Prochnow et al. 2006, Zhang and Fang 2007), and form of available nitrogen (Rygiewicz et al. 1984a,b, Brix et al. 2002), as well as being associated with soil salinity effects (Yousfi et al. 2007), the effect of pH itself on tree physiology has not been studied to the same extent. However, a couple of studies to date have shown that pH can be a factor in root water relations (Tang et al. 1993, Kamaluddin and Zwiazek 2004).

Ectomycorrhizal (ECM) fungi have been shown in several experiments to help plants mitigate the effects of environmental stresses such as low pH conditions associated with acid rain (Honrubia and Diaz 1996, Cordell 1997), mining operations (review by Colpaert and Van Assche 1987, Jentschke and Godbold 2000), and liming of soil (Aggangan et al. 1996, Wallander 2002). These studies have reported improved reforestation, overall tree survival, increased nutrient acquisition (reviewed in Cordell et al. 2000), and lower levels of heavy metals (Marx and Artman 1979, Jentschke et al.

1991, Bücking and Heyser 1994) in mycorrhizal plants. Plants inoculated with mycorrhizae have been used in land reclamation and remediation projects involving revegetation of acidic (Cordell et al. 1991) and alkaline (Kernaghan et al. 2002) soils, and mine tailings (Moynahan et al. 2002, Withers 2002), including Alberta oilsands (Bois et al. 2005, Khasa et al. 2005). However, some studies have also documented minimal or no increase in plant growth and survival as a result of mycorrhizal associations when plants were exposed to naturally-occurring or artificially-imposed environmental stresses (Cripps 2001b). To date, no research has attempted to understand the underlying mechanisms by which mycorrhizal fungi may improve plant growth at these physiological pH extremes.

Trembling aspen (*Populus tremuloides*) is a commercially-important (Perala and Carpenter 1985, Peterson and Peterson 1992), relatively fast-growing boreal and temperate forest tree species. Its high adaptability to environmental changes (Howard 1996) and fast vegetative regeneration on disturbed sites has made it suitable for land reclamation and revegetation of acidic strip mine sites (Hughes 1990, Winterhalder 1990), mine spoils (Schier et al. 1985), and riparian restoration projects (Chan and Wong 1989). Growth of aspen and other deciduous boreal tree species tends to be more limited on acidic, low-nutrient soils, which favour the growth of conifers (Jerabkova et al. 2006, Westbrook et al. 2006), although it is unclear whether it is the low nutrient availability or other factors that are the main limiting factors for aspen growth. Ectomycorrhizal (ECM) fungi such as *Hebeloma crustuliniforme*, an alkaliphilic ECM fungus with a wide boreal forest distribution and commonly associated with trembling aspen (Hutchison 1991), may enable its establishment and increase its growth in these pH-affected areas (Shuffstall and Medve 1979, Cripps 2003).

The objectives of this present study were to: 1) examine the short-term effects of different pH on trembling aspen root systems inoculated with *H. crustuliniforme*, and; 2) examine the effect of pH on longer-term growth of *H. crustuliniforme* in pure culture with different forms of supplied inorganic nitrogen. In this study, a short-term seedling experiment was carried out, since short- and long-term mechanisms of adaptability to root water transport may differ. Because trembling aspen is a highly-adaptable tree species, a short-term experiment may provide more insight into short-term regulation of root water flux in response to pH, since a long-term experiment would likely also involve changes in root anatomy that would ultimately affect root water transport. It was predicted that: 1) due to of the alkaliphilic nature of *H. crustuliniforme*, mycorrhizal aspen would show an alkaline-directed pH shift in their pH optimum for root water relations parameters compared with non-mycorrhizal seedlings; 2) *H. crustuliniforme* in pure culture would demonstrate an alkaline pH growth optimum that would be affected by the form of supplied inorganic nitrogen, and; 3) *H. crustuliniforme* would demonstrate some ability to modify the pH of its growth medium.

4.2. MATERIALS AND METHODS

4.2.1. Mycorrhizal culture

Hebeloma crustuliniforme (Bull.) Quél. (University of Alberta, Devonian Microfungus Collection, UAMH 5247) was sub-cultured on Melin Norkrans Media (MNM) agar (Mason 1980). Plugs of MNM agar-grown fungus were aseptically sub-cultured in MNM liquid media and placed on an orbital shaker in aerated flasks for 4 weeks to produce liquid culture. Mycelia grown in liquid culture was homogenized under sterile conditions using a blender to produce liquid inoculum for seedling roots.

Plugs of MNM agar-grown fungi were sub-cultured on plates of Murashige and Skoog (MS) agar media (Murashige and Skoog 1962), modified by decreasing the sucrose concentration (3 g L^{-1}) to create a low-sucrose media for pure culture fungal growth. MS media, although typically used for growth of plant cell cultures, was used in this experiment since the author had successfully cultivated mycorrhizal seedlings in aseptic culture previously for anatomical examination of mycorrhizal root colonization using low-sucrose MS media. *H. crustuliniforme* was grown on low-sucrose MS media for approximately 4 weeks prior to transfer to pH treatment plates.

4.2.2. Plant culture

Populus tremuloides Michx. seeds were collected from the North Saskatchewan river valley (Edmonton, Alberta, Canada) in 2004 and stored at -10°C until use. Seeds were removed from storage, surface-sterilized with 5% sodium hypochlorite solution for five minutes, and rinsed thoroughly with deionized water. Seeds were germinated on washed, sterile silica sand in Petri dishes for up to five days, then transplanted to styroblocks™ (superblock 160/60, Beaver Plastics Ltd., Edmonton, AB, Canada) filled with sterile 1:1 peat:sand mixture. Germinants were placed in a controlled-environment growth chamber (60% RH, 18 hr photoperiod, 22°C/18°C day/night, 300 µmol m^{-2} s^{-1} PAR, full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada)). Seedlings were bottom-watered every other day with deionized water, and once weekly with 0.1% 20-20-20 fertilizer solution (Plant Prod® Water-Soluble Fertilizer, Spectrum Brands Inc.).

After six weeks, seedlings were transferred to 12 cm diameter pots containing a 2:1:1 peat-perlite-sand mixture. The bottoms of the pots were lined with a layer of aluminum foil and placed inside a second pot to prevent flow-through during watering. Following transplantation, fertilizer solution was omitted from the watering schedule for three weeks to induce nutrient deficiency in preparation for mycorrhizal inoculation.

4.2.3. Mycorrhizal inoculation of aspen seedlings

Half of the seedlings were inoculated with a total of 5 mL of *H. crustuliniforme* homogenized liquid medium. The medium was injected with a wide-tip sterile pipette at least 3 cm below the soil surface in two locations near the base of the seedling stems. The other half of the seedlings were designated as the non-mycorrhizal (control) group and were inoculated with 5 mL of autoclaved, fungal-free, liquid MNM, applied to control seedlings in the same manner as for mycorrhizal inoculation. Mycorrhizal and non-mycorrhizal seedlings were separated from each other in the growth chamber to prevent contamination of non-mycorrhizal seedlings with the fungal inoculum introduced to mycorrhizal seedlings. Following inoculation, seedlings were watered with a small amount of water every other day for two weeks. At the start of the third week, 0.025%

20-20-20 fertilizer solution (Plant Prod® Water-Soluble Fertilizer, Spectrum Brands Inc.) was applied twice per week as part of the regular watering schedule. Seedlings were grown for an additional 9 weeks following inoculation to ensure adequate colonization prior to the start of pH treatments.

4.2.4. pH treatment of aspen seedlings and measurements

4.2.4.1. pH treatment of aspen seedlings

Seedling root systems were removed from pots prior to application of pH treatments, and gently rinsed with deionized water to remove as much potting medium as possible without damaging roots. Seedling roots were detopped with a razor blade, leaving approximately 2 cm of stem attached to root systems. Roots were immersed in an aqueous root bathing medium of 0.02% trisodium 3-hydroxy-5,8,10-pyrenetrisulfonic acid (PTS_3), a fluorescent tracer dye restricted to the apoplastic pathway of water movement (discussed by Steudle and Peterson 1998) that has been used to measure relative changes in water transport through the apoplast (Siemens and Zwiazek 2003, Schaider et al. 2006). After roots were immersed, the immersion solution was pH-adjusted to one of the six pH treatments (4, 5, 6, 7, 8, 9) with either 1 M H_2SO_4 or KOH, while continuously being stirred with a magnetic stirrer, until pH of the root immersion solution stabilized (target pH $\pm$ 0.1 unit). All pH treatments were applied in this manner to both mycorrhizal and non-mycorrhizal seedlings.

4.2.4.2. Root water relations and xylem exudate measurements of aspen seedlings

Roots in their pH-treatment media were placed in Scholander pressure chambers (PMS Instruments, Corvallis, OR). The root bathing solution was continuously aerated during measurements with a magnetic stir bar using a magnetic stirrer placed underneath each pressure chamber. Root water flow (Q_v, $m^3\ s^{-1}$) was measured for a minimum of 20 minutes at hydrostatic pressures of 0.4, 0.6, 0.8, and 1.0 MPa, a range within which changes in Q_v are known to be linear with pressure changes in aspen (Siemens and Zwiazek 2003, 2004). A minimum of 10 minute intervals between Q_v measurements at each increasing pressure were maintained to stabilize Q_v values. Once initial root water flux (J_v, m^3 s^{-1} MPa^{-1}) measurements were made, root systems were de-pressurized for opening of the pressure chambers. $HgCl_2$ solution was added to the PTS_3 solutions inside the pressure chambers to reach a final concentration of 0.10 mM and the pH of the solutions were readjusted back to their target pH. Root systems were re-pressurized for 30 minutes at 0.4 MPa, following which a second set of J_v values was collected at the same four hydrostatic pressures. $HgCl_2$ has been reported to physically and reversibly restrict the passage of water through aquaporins (AQPs), transmembrane water channel proteins found in all plant tissues including roots, by binding to sulfhydryl groups of cysteine residues near the apoplastic surface of cells (Siefritz et al. 2002, Lovisolo and Schubert 2006).

Xylem exudate samples were collected from each detopped roots under pressure following both sets of J_v measurements for measurement of xylem PTS_3 concentrations. Samples were diluted with water and measured for PTS_3 concentration against pH-adjusted PTS_3 standard curves using a Sequoia-Turner 450 spectrofluorometer (Apple Scientific, Chesterland, OH, USA) with a 405 nm excitation and 515 nm emission spectrum (Skinner and Radin 1994).

Root volumes were measured for each root system using volume displacement of water in a graduated cylinder (Voicu and Zwiazek 2004). Root volumes were used to calculate L_{pr} (s^{-1} MPa^{-1}).

4.2.5. Nitrogen and pH treatments for H. crustuliniforme and growth measurements

4.2.5.1. Nitrogen and pH treatments of H. crustuliniforme culture

Four nitrogen treatments and six pH treatments were used, for a total of 24 nitrogen and pH treatment combinations. The four nitrogen treatments were no inorganic nitrogen (O), nitrate only (N), ammonium only (A), and nitrate + ammonium (NA). 8 mM total inorganic nitrogen was supplied in each of the N, A, and NA treatments. The six pH treatments were pH 4, 5, 6, 7, 8, and 9. To apply the treatments, modified MS media (Murashige and Skoog 1962) was used, with noted modifications. Low sucrose concentrations were supplied (4.7 g L^{-1} for N, A, NA) to ensure an optimal 20:1 C:N ratio for all treatments containing inorganic nitrogen. A minimal amount of sucrose, 0.12 g L^{-1}, was supplied in the modified MS media for O, to provide a minimal amount of sucrose in the absence of inorganic nitrogen, which resulted in a C:N ratio of 37:1.

To control all unquantifiable forms of available carbon and nitrogen in MS media, casein enzyme hydrolysate was omitted from the media preparation, although the small concentration of organic N-containing vitamins (nicotinic acid, thiamine-HCl, pyridoxine-HCl) that are part of the MS formula were added to the media. In the inorganic nitrogen treatments, nitrate was supplied as $Ca(NO_3)_2$ and KNO_3, ammonium was supplied as $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$, and nitrate and ammonium, when supplied together, were provided as NH_4NO_3. Purified agar (cat. # A1296, Sigma-Aldrich Canada) was used as the gelling agent for all plates. Although there was no available report for trace element concentration of nitrogen in the purified agar used, it was assumed that it was low (<1%) based on typical trace element concentrations of other macronutrients.

Because the pH of growth media changes during autoclaving, and because agar media solidification varies with pH, a few preliminary experiments were conducted to determine the pH adjustments and quantity of agar that needed to be added to each treatment prior to autoclaving to obtain the proper pH and media solidification after autoclaving, and to ensure that the media in each treatment had approximately equivalent solidification. Agar powder added to media varied between 22 g L^{-1} (pH 4) to 8 g L^{-1} (pH 9). pH adjustment prior to autoclaving was dependent on the nitrogen treatment. All pH measurements were taken with a flat-surface electrode (Cat. # 13620289, Fisher Scientific Co., Ottawa, Canada). Pre- and approximate post-autoclaving with pH values for the four nitrogen treatments (O, N, A, NA) are listed below.

Target pH	pH before autoclaving	pH after autoclaving
4	$3.95^{O}, 3.97^{N}, 4.00^{A}, 4.00^{NA}$	$3.92^{O}, 4.11^{N}, 4.04^{A}, 3.97^{NA}$
5	$5.10^{O}, 5.05^{N}, 5.14^{A}, 5.13^{NA}$	$5.10^{O}, 4.83^{N}, 4.94^{A}, 4.98^{NA}$
6	$6.60^{O}, 7.20^{N}, 6.75^{A}, 6.53^{NA}$	$6.25^{O}, 5.82^{N}, 6.08^{A}, 6.08^{NA}$
7	$7.60^{O}, 8.00^{N}, 7.75^{A}, 7.93^{NA}$	$7.05^{O}, 7.27^{N}, 7.12^{A}, 7.28^{NA}$
8	$9.46^{O}, 9.86^{N}, 9.06^{A}, 9.32^{NA}$	$7.96^{O}, 8.00^{N}, 8.01^{A}, 8.14^{NA}$
9	$11.2^{O}, 11.45^{N}, 10.85^{A}, 11.05^{NA}$	$9.25^{O}, 9.10^{N}, 8.78^{A}, 8.86^{NA}$

A minimum of eight (n=8) 100 mm diameter Petri dishes, each containing approximately 30 mL of autoclaved modified low-sucrose MS media, were poured for each pH*nitrogen treatment combination. Pieces of porous cellophane (Cat. # 361004007, Fisher Scientific Co., Ottawa, Canada) were trimmed, autoclaved separately, and aseptically transferred to the autoclaved, solidified plates of media. Cellophane circles were laid on top of the solidified media of each dish to facilitate harvesting of intact colonies at the end of the experiment. One 8 mm diameter plug of low-sucrose MS-grown *H. crustuliniforme*, cut from the periphery of an existing colony, was aseptically transferred to each pH*nitrogen treatment Petri dish, and centered in the middle of each piece of cellophane. Petri dishes were then sealed with Parafilm and stored upright. Two days after completion of mycelial transfers to all pH*nitrogen treatment plates, they were inverted and incubated at room temperature for 6 weeks.

4.2.5.2. Growth measurements of H. crustuliniforme culture

Colony diameters were measured by taking two diameter measurements through the center of the colony at intersecting 90° angles. Colonies and their underlying cellophane circles were removed and fresh weight of each colony was measured, minus the weight of the cellophane circles.

Once colonies were removed, pH measurements were taken of the agar media on which fungal colonies had been growing. For each plate, two pH measurements were taken: one at the outer edge of the agar where no mycelia were present; and in the center of the agar which was directly underneath the center of the colony. Both pH values for each plate were averaged and used as the final pH value of the agar following mycelial growth on the agar.

4.2.6. Statistical analysis

All data were analyzed with SAS 9.1. (SAS Institute, North Carolina, USA) to determine statistically significant ($p \leq 0.05$) differences between treatment combinations of pH level and mycorrhiza for aspen, and for differences between treatment combinations of pH level and nitrogen form for H. *crustuliniforme*. All data were first tested for normality of distribution and homogeneity of variance. Residuals from the data sets were calculated and used to graph normal probability plots and box plots, to determine if there were any statistical outlier values in the data sets. Outliers, if any, were removed from the data sets.

Measurements (n=10) from mycorrhizal and non-mycorrhizal seedlings (as described in section 4.2.4.2.) were analyzed using a MIXED analysis of variance (ANOVA) randomized complete block (RCB) design with a 6 (pH) x 2 (mycorrhizae) factorial model to statistically compare differences in the means for each mycorrhizal and pH treatment combination. A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment group harvested per day. Therefore, the days of measurements were the blocks. The statistical model used for the analysis was:

$Y_{ijkl} = u + M_i + P_j + M_iP_j + B_k + e_{ijkl}$, where

Y_{ijkl} = value of individual observation (i=mycorrhiza, j=pH, k=block, l=observation)

u = overall mean of observations

M_i = effect of i^{th} treatment (i = mycorrhizal treatment)

P_j = effect of j^{th} treatment (j = pH level)

M_iP_j = interaction effect of i^{th} and j^{th} treatments

B_k = effect of k^{th} block (k=day of measurement)

e_{ijkl} = residual error

Measurements (n=8) from pure culture plates of *H. crustuliniforme* (as described in section 4.2.5.2.) were analyzed using a 4 (nitrogen form) x 6 (pH) factorial complete randomized (CR) design ANOVA model to compare measurement means for each combination of mycorrhizal and pH treatment. The statistical model used for the analysis was:

$Y_{ijk} = u + N_i + P_j + N_iP_j + e_{ijk}$, where:

Y_{ijk} = value of individual observation (i=nitrogen, j=pH, k=observation) u = overall mean of observations

N_i = effect of i^{th} treatment (i = nitrogen form)

P_j = effect of j^{th} treatment (j = pH level)

N_iP_j = interaction effect between i^{th} and j^{th} treatments

e_{ijk} = residual error

Comparisons from all the ANOVA tests were conducted using least-squares means. For MIXED ANOVA, corresponding Tukey-adjusted p-values were used to further examine differences among the treatment combinations using pre-planned comparisons (α=0.05). The slice function was used to analyze and interpret interactions between main effects that were statistically significant (α=0.05) for multi-factor MIXED ANOVA. The results of all statistical analyses are presented in figures and tables at the end of this chapter.

4.3. RESULTS

4.3.1. Root hydraulic conductivity

Mean initial L_{pr} values of non-mycorrhizal (CTRL) seedlings prior to the addition of $HgCl_2$ did not change significantly with short-term changes in pH, and ranged between 1.24×10^{-4} s^{-1} MPa^{-1} (pH 4) and 1.65×10^{-4} s^{-1} MPa^{-1} (pH 5) (Fig. 4.1A). In contrast, L_{pr} of mycorrhizal (MYCO) seedlings increased significantly ($p<0.05$) in response to increasing pH from 4-7, with a sharp decline in L_{pr} at pH 8-9. Only at pH 7 were L_{pr} means of MYCO and CTRL seedlings significantly different.

Following the addition of $HgCl_2$ to roots, normalized L_{pr} decreased for all of the pH treatments in CTRL and MYCO seedlings, except for MYCO seedlings at pH 8. Normalized L_{pr} was calculated as a percentage of the L_{pr} values prior to the addition of $HgCl_2$ to roots, which are represented by the line at 100% (Fig. 4.1B). Normalized L_{pr} of CTRL seedlings did not change significantly with pH treatment. In comparison, normalized L_{pr} of MYCO seedlings increased significantly with increasing pH. Only at pH 8 was normalized L_{pr} significantly different between MYCO and CTRL seedlings.

Although there were no significant statistical interaction effects (mycorrhizae*pH) for L_{pr} or normalized L_{pr}, the main effect of pH was highly significant ($p \leq 0.001$) for normalized L_{pr}. The main effect of mycorrhizae was also significant ($p \leq 0.05$) for normalized L_{pr} (data not shown).

4.3.2. PTS₃ concentrations in xylem exudate

PTS_3 concentrations in xylem exudate prior to the addition of $HgCl_2$ changed significantly with pH treatment in CTRL seedlings, but not in MYCO seedlings (Fig. 4.2A). Means for CTRL seedlings ranged from 2.64 x 10^{-3} % (pH 6) to 3.72 x 10^{-3} % (pH 4), with a trend of higher PTS_3 means at pH 4 and 9, and significantly lower means for the intermediate pH treatments (5-8). In comparison, PTS_3 means for MYCO seedlings showed a slight non-significant decrease from pH 4 to 9. Only at pH 9 was there a significant difference between CTRL and MYCO PTS_3 means.

Following the addition of $HgCl_2$ to roots, PTS_3 concentrations increased in all treatments except for pH 9 (CTRL, MYCO seedlings) and pH 8 (MYCO seedlings). This relative increase is shown as normalized PTS_3 concentration (Fig. 4.2B), and was calculated as the relative increase in PTS_3 concentrations following $HgCl_2$ addition compared to PTS_3 concentrations prior to the addition of $HgCl_2$ (indicated by the line at 100%). Normalized PTS_3 decreased significantly with increasing pH in both CTRL and MYCO seedlings, although the decrease was greater for CTRL than for MYCO seedlings. At pH 4, the normalized PTS_3 mean for CTRL seedlings was significantly higher than that of MYCO seedlings.

There were no statistically significant interaction effects (pH*mycorrhizae) for PTS_3 or normalized PTS_3 measurements, although the main effect of pH was highly significant ($p \leq 0.001$) for normalized PTS_3. The main effect of mycorrhizae was not significant for either PTS_3 or normalized PTS_3 (data not shown).

4.3.3. Fresh weight and diameter of H. crustuliniforme colonies

Fresh weight means of the colonies significantly increased with increasing pH for the no nitrogen (O), nitrate+ammonium (NA), nitrate (N), and ammonium (A) treatments, although the extent of the increases across the pH range differed between the nitrogen treatments (Fig. 4.3). For N and A, the lowest colony growth occurred at pH 5 and 6, respectively, whereas for O and NA, the lowest colony growth occurred at pH 4. With respect to changes in fresh weight across the pH range, treatment A was most affected by pH (0.124 g difference), and NA treatment was least affected by pH (0.058 g difference). Maximum colony fresh weight occurred at pH 9 (O, N, A) and pH 8 (NA), with N having the highest colony fresh weight of all four nitrogen treatments.

At each pH, differences in colony fresh weight were significant between nitrogen treatments. At pH 4, 5, and 7, O had the highest and NA had the lowest fresh weights. At pH 6 and 8, O had the highest, and A had the lowest fresh weights. At pH 9, N had the highest and NA had the lowest fresh weights. These differences between all nitrogen treatments were least significant at pH 7 and 8.

Changes in colony diameter in response to pH and nitrogen treatments (Fig. 4.4) were similar to those of colony fresh weight. The effect of pH on colony diameter differed between nitrogen treatments, although colony diameter generally increased significantly with increasing pH (4-8) for NA, N, and A treatments, and significantly decreased at pH 9. Changes in colony diameter with pH for the O treatment were slightly different, in comparison, and showed significant increases from pH 4-5 and 7-8, with a decrease from pH 6-7 and from pH 8-9.

For all nitrogen treatments, the smallest colony sizes occurred at pH 9, and largest sizes occurred at pH 5 (O), 6 (N, A), and 7 (NA). With respect to changes in colony diameter across the pH range, N treatment was most affected (difference of 4.44 cm), and NA treatment was least affected by pH (3.97 cm difference). N treatment also had highest maximum colony diameter of all four nitrogen treatments. As with colony fresh weight, differences in pH changes between all nitrogen treatments at each pH were least significant at pH 7 and 8.

4.3.4. Nutrient agar pH changes due to growth of H. crustuliniforme

Growth of *H. crustuliniforme* (Hc) on the nutrient agar affected the pH of the agar media. Due to fungal growth on the nutrient agar, the agar pH changed significantly from the initial pH for many of the pH treatments, but the extent of the difference depended upon the form of nitrogen (Fig. 4.5). These changes were significantly different between pH treatments within each nitrogen treatment, and between nitrogen treatments for the same pH treatment. Hc generally resulted in a decrease in agar pH for most of the nitrogen and pH treatment combinations, except for a few pH treatments within the O and N nitrogen treatments. The greatest pH changes due to mycorrhizal growth were at pH 9 for O (-0.621) and N (-0.464), and at pH 8 for NA (-0.248) and A (-0.424). The smallest pH changes due to mycorrhizal growth were at pH 8 for O (+0.0144), pH 7 for A (-0.05), and at pH 4 for NA (-0.0238) and N (+0.00688). These differences did not represent significant changes in pH. For NA and N treatments, the least pH changes occurred at pH 4. For O and A treatments, the smallest pH changes occurred between pH 6-7.

The statistical main effects (nitrogen, pH) and interactions (nitrogen*pH) for all measurements of *H. crustuliniforme* were highly significant ($p<0.0001$) for mean colony fresh weight, mean colony diameter, and mean pH changes (Tab. 4.1). The slice function in SAS (slice=nitrogen) determined that for each nitrogen treatment (A, N, NA, O), the changes in response variables were highly significant ($p<0.0001$) with the differences in pH treatments.

4.4. DISCUSSION

In the present study, it was apparent that the short-term effects of pH alone affected MYCO seedlings to a greater extent than CTRL seedlings. MYCO seedlings had higher L_{pr} at alkaline (> 7) pH, compared with CTRL seedlings. L_{pr} was not affected by pH in CTRL seedlings. Increased L_{pr} in MYCO seedlings is consistent with previous studies that have reported increased root water flux in ectomycorrhizal plants (Muhsin and Zwiazek 2002b, Bogeat-Triboulot et al. 2004, Marjanović et al. 2005). This can occur through increased symplastic (Muhsin and Zwiazek 2002a) as well as apoplastic water flux (Landhäusser et al. 2002) created by hyphal root extensions (Smith and Read 1997). The results of the present study seem to indicate two separate findings: 1) mycorrhizal associations (in this case, between trembling aspen and Hc) may increase root water transport; 2) Hc, an alkaliphilic fungus, may be somewhat capable of conferring alkaline tolerance to its plant host.

The fact that short-term pH changes did not substantially affect root L_{pr} in CTRL seedlings was unexpected, considering that *Populus* tends to grow in temperate boreal forest soils with a pH range of 4.0-5.6 (Vitousek et al. 1982, Jerabkova et al. 2006). Although this pH range suggests that aspen does not prefer alkaline conditions, this does not necessarily mean that aspen is alkaline-intolerant. It is not currently known to what extent aspen is tolerant of alkaline pH conditions in a natural environment, although a previous study reported *Populus* did not grow well in alkaline pH conditions when ammonium nitrate fertilization was provided (DesRochers et al. 2006). Other studies have shown reductions in root water flux in several plant species with high (Tang et al. 1993, Ktitorova et al. 1998, Kamaluddin and Zwiazek 2004), and low (Gunsé et al. 1997, Kamaluddin and Zwiazek 2004) pH. However, most previous studies did not examine immediate pH effects, and therefore little is known about the primary mechanisms of root response to initial pH exposure. In studies with pH exposure ≥18 h, reductions in root water flux may have been largely due to reduced plant growth (Tang et al. 1993), reduced root growth (Gunsé et al. 1997), plasmodesmatal closure (Ktitorova et al. 1998), or pH-mediated ABA-induced stomatal closure (Kamaluddin and Zwiazek 2004). With more immediate pH effects, reduced root water flux at low and high pH may be due to decreased AQP activity (Kamaluddin and Zwiazek 2004). It is possible that, in the present study, AQP activity may have been a factor in the alteration of L_{pr} in response to pH.

In the present study, pH seemed to have an effect on AQP activity in aspen seedlings, although the effect was more pronounced in MYCO compared with CTRL seedlings. Activity of Hg-responsive AQPs, indirectly measured by the relative changes in L_{pr} and xylem exudate PTS_3 concentrations following the addition of $HgCl_2$ to root systems, indicated that AQP activity was high at low pH (4) and lower at pH >7. Although there has been no published research to date on root or mycorrhizal AQP regulation in response to environmental pH changes, pH may also have a direct affect on root AQPs, which extensively mediate the cell-to-cell pathway for root water flux (Voicu and Zwiazek 2004, Aroca et al. 2006). Additionally, it has been reported that plasma membrane intrinsic protein (PIP) AQP activity can be regulated via cytosolic pH changes that occur in response to anoxia and drought stress. Both Tournaire-Roux et al. (2003) and Pettersson et al. (2006) demonstrated that AQP-mediated root water flux in plants and AQP activity in yeast, respectively, decreased at acidic pH values. Alleva et al. (2006) also found that acidification below pH 6.6 resulted in reduced AQP-mediated flow compared with higher cytosolic pH (8.3) in purified membrane vesicles. This pH-induced regulation is due to a pH-sensitive gating mechanism that results in protonation of a histidine (His) residue located within a highly-conserved PIP region under acidic conditions, which in turn produces a conformational change with closure of the pore on the cytosolic side (Törnroth-Horsefield at al. 2006). His residues are considered to be primary sites of pH regulation because His has a pKa of ~ 6.5 (Zelenina et al. 2003).

In the current study, L_{pr} significantly increased as pH increased in MYCO seedlings, but this was inversely related to AQP activity. This was in contrast with previous studies where acidic pH decreased both root water flux and AQP-mediated activity (Tournaire-Roux et al. 2003, Alleva et al. 2006, Pettersson et al. 2006). This could mean that AQP activity had little effect on overall L_{pr}, such as in CTRL seedlings where pH had little effect on L_{pr} despite pH-induced reduction in AQP activity at pH 7. However, in MYCO seedlings, there was a greater effect of pH on both L_{pr} and AQP activity, despite the inverse relationship. One explanation for this could be that that pH can affect membrane lipid composition (Palmgren et al. 1988) which may then alter AQP conformational structure so that AQPs can develop either an Hg-responsive or Hg-unresponsive configuration. Therefore, the observed reduction in AQP activity with increasing pH in the present study may simply have been a reduction in Hg-responsive AQP activity, rather than all AQP activity. A second explanation could be the fact that aspen may have a slightly different pH optimum for AQP function because of its adaptations to acidic forest soils, unlike crop species which tend to grow in agricultural soils with a higher pH (6.0-8.0) (Canadian Council of Ministers of the Environment 1999), and which were the subject of the previous AQP pH studies (Tournaire-Roux et al. 2003, Alleva at al. 2006, Pettersson et al. 2006). The possibility of a different pH optimum for MYCO aspen AQPs is also supported by animal AQP pH studies, which demonstrated that the position and number of multiple His residues can modulate pH sensitivity to a more acid or alkaline range (Németh-Cahalan et al. 2004). Extracellular pH and the involvement of both His and serine (Ser) residues have resulted in reduced AQP activity in human cells (Zelenina et al. 2003). Additionally, activity corresponded to typical *in situ* physiological pH, with minimum AQP activity at pH ~ 7 and higher activity at pH 4 (Yasui et al. 1999, Németh-Cahalan and Hall 2000).

The fact that Hc, an alkaliphilic fungus (Hung and Trappe 1983, Kernaghan et al. 2002), seemed to result in a shift towards maximum L_{pr} within an alkaline pH range in MYCO seedlings, may have been due to pH preference of the fungus. To the author's knowledge, nothing is currently known about AQP function or activity in ECM mycelia, particularly in response to pH. However, the present study did demonstrate that Hc had a preference for an alkaline growth medium as expected, indicated by greater growth at pH >7, although the effect of pH on fungal growth was somewhat modified by the form of nitrogen. This was observed particularly with colony fresh weight, and to a lesser extent with colony diameter. Although colony fresh weight continued to increase with pH for all nitrogen treatments, colony diameter peaked between pH 6-8, indicating differences in density and growth patterns that were dependent upon the form of nitrogen as well as pH (indicated by the significant statistical interaction effects in Tab. 4.1). The data indicated that Hc preferred nitrate (N) overall, especially at pH >7, although Hc seemed to prefer either ammonium (A) or nitrate + ammonium (NA) at lower pH (4-5). Hc had slightly greater colony diameter when grown on A and slightly higher fresh weight when grown with NA at pH >7, suggesting that Hc does not appear to have a preference for either A or NA. It was expected that Hc would show a slight preference for nitrate, although Hc can also utilize ammonium (Quoreshi et al. 1995). It was also expected that growth of ECM fungi would be affected by external pH, as reported previously (Wallander 2002).

The alkaline but not acid tolerance of Hc may have been part of the reason for the increased MYCO L_{pr} at pH >7 and reduced L_{pr} at pH 4-5. Additionally, Hc may have increased the alkaline tolerance of associated aspen because of its modest ability to modify the pH of its environment. Mycorrhizal roots, in response to different forms of supplied nitrogen, may be better able to buffer the pH of the rhizosphere via H^+/OH^- flux compared with non-mycorrhizal roots (Rygiewicz et al. 1984a, Rigou et al. 1995, Bago and Azcon-Aguilar 1997). However, it is not clear how root-associated mycorrhizal hyphae may contribute to this ability. In the present study, it was apparent that Hc could modify the pH of its growth media in pure culture, but the extent of modification was dependent upon both the supplied form of nitrogen and the initial pH. With N and O, pH of the media increased at lower pH (4-6), and with A and NA, pH of the media decreased at pH 4-6. This was likely due to the mechanisms of alkalinization and acidification, respectively, of the media previously observed in mycorrhizal roots (Rygiewicz et al. 1984a,b, Bago and Azcon-Aguilar 1997). Media that had a higher initial pH showed larger decreases after the growth of Hc, regardless of nitrogen form, although O and N had the larger decreases of all four nitrogen treatments. These findings are consistent with previous studies which demonstrated that mycorrhizae were capable of modifying the pH of their growth substrate, and that increases or decreases in pH were also dependent on nitrogen form supplied (Zhu et al. 1994, Quoreshi et al. 1995). While the pH changes due to mycorrhizal growth at pH 9 were not large (differences of 0.1-0.6 pH), they do suggest that Hc is capable of slightly lowering the pH of its media in an alkaline environment. pH modification by fungi may be of physiological importance for host plants if a slight pH decrease results in increased root water flux at pH >7, and may have implications in conferring alkaline tolerance to host plants. However, it is not clear from the present study if pH modification by Hc had any effect on the L_{pr} of MYCO seedlings. Certainly, the role of rhizospheric pH modification by hyphal tissue merits further study.

Because the mechanics of root water flux in ECM associations are poorly understood, it is possible that the influence of Hc on MYCO seedlings may not be limited to pH modification of the growth media. Mycorrhizal fungi have been shown to influence AQP expression in plant roots due to symbiosis (Frettinger et al. 2007) and in response to environmental stress such as drought (Uehlein et al. 2007). AQPs are known to be present in yeast (Soveral et al. 2006), although they have not as yet been reported in mycorrhizal fungi. If pH-sensitive AQPs were present in Hc tissue, then AQP activity in Hc may have been higher at alkaline pH, reducing resistance to root water flux through hyphal tissue at pH >7 in the present study. Additionally, mycorrhizae may have more of an effect on apoplastic flow rather than AQP-mediated transport, as reported in another study where an inverse relationship between AQP activity and L_{pr} in mycorrhizal tree roots was also observed (Muhsin and Zwiazek 2002a). pH may have other effects on the association between fungus and plant, such as altering the extent of root colonization by fungi (Medeiros et al. 1994), and altering mycorrhizal structures associated with roots (Wilcox and Wang 1987). Structural modifications or non-AQP differential resistances in fungal-root associations can lessen or increase resistance to water flux through tighter or looser mantle construction (Bogeat-Triboulot et al. 2004). However, water and ionic movement through ECM fungal tissue and between the fungus and the root is currently not understood, especially in angiosperms (Peterson at al. 2004).

4.4.1. Conclusions

Hc showed a preference for higher pH as indicated by increased colony diameter and fresh weight. Its growth response to pH was affected by the form of nitrogen, showing a slight preference for nitrate over ammonium or a combination of nitrate and ammonium. Hc was also capable of modifying the pH of its growth medium in pure culture, by lowering the pH (difference of 0.1 to 0.6 pH units) at pH 9. Although this pH modification was relatively small, it may have some physiological significance to associated plants if, by lowering the rhizospheric pH, the end result is a slight increase in nutrient solubility or L_{pr} for host plants.

Although trembling aspen is adapted to more acidic forest soil conditions, it was observed that aspen was able to tolerate short-term alkaline conditions. L_{pr} was not affected by pH in CTRL seedlings, but MYCO seedlings displayed a maximum L_{pr} at pH >7, possibly because of the preference for alkaline pH that was shown by Hc in pure culture. AQP activity in both CTRL and MYCO seedlings decreased with increasing pH, which was inversely related to L_{pr}. Therefore, changes in L_{pr} may not have been due to AQP regulation, particularly in MYCO seedlings.

Hc resulted in increased L_{pr} of MYCO aspen, and may also have increased aspen's alkaline tolerance because of Hc's increased growth under alkaline conditions and its slight ability to modify the pH of its environment. The results of this study suggest that trembling aspen, which is currently being studied for use in oilsands land reclamation projects (Khasa et al. 2002, 2005) and Hc, which grows well in culture and tree nurseries (Cripps 2001a, Marmeisse et al. 2004) both have traits that may make them suitable for long-term use in alkaline land reclamation and reforestation projects. Continued research is needed to understand the interactions between ECM fungi and plant roots, and the mechanisms of changes in water flux in mycorrhizal roots, particularly in response to environmental pH.

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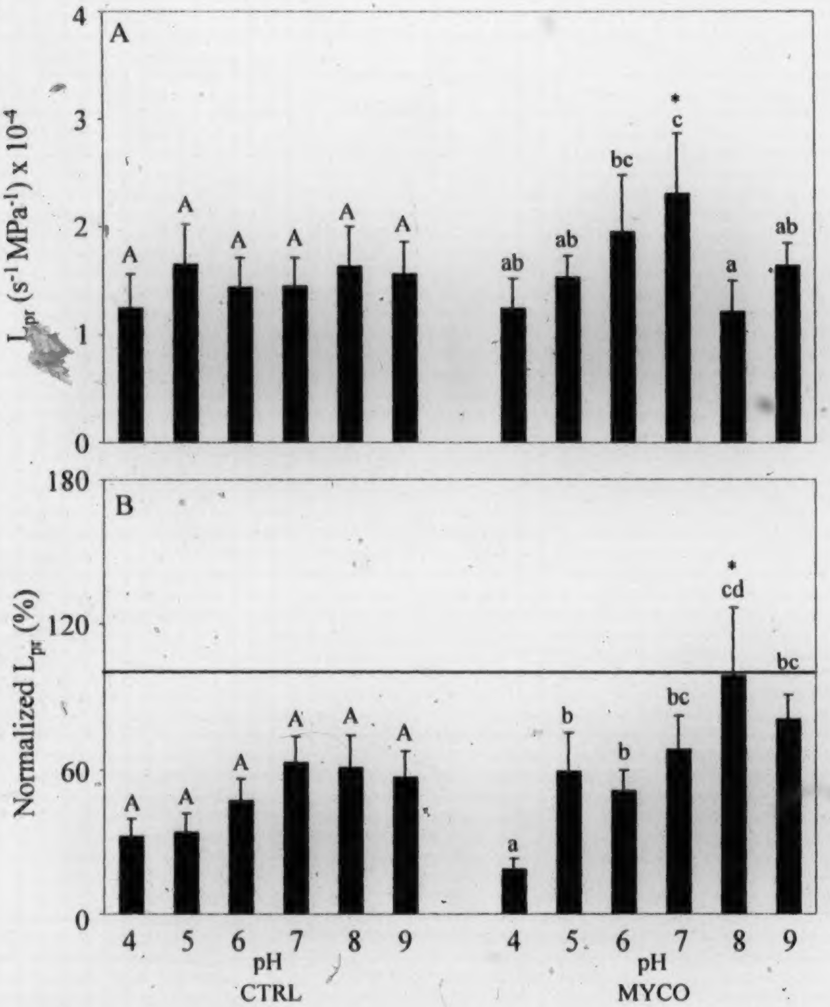

Figure 4.1. (**A**) Root hydraulic conductivity (L_{pr}) measured prior to the addition of $HgCl_2$. (**B**) Normalized L_{pr} measured after the addition of 100 μM $HgCl_2$ and calculated as a percentage of untreated L_{pr} values (shown in **Fig. 4.1A**, and indicated by the line at 100% in **Fig. 4.1B**), in non-mycorrhizal (CTRL) or mycorrhizal (MYCO) aspen seedlings receiving a short-term pH treatment (pH 4-9). Least-squares means ± SE are shown (n=10). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among pH treatments within CTRL seedlings), lowercase letters (among pH treatments within MYCO seedlings) and asterisks (between CTRL and MYCO for a given pH treatment).

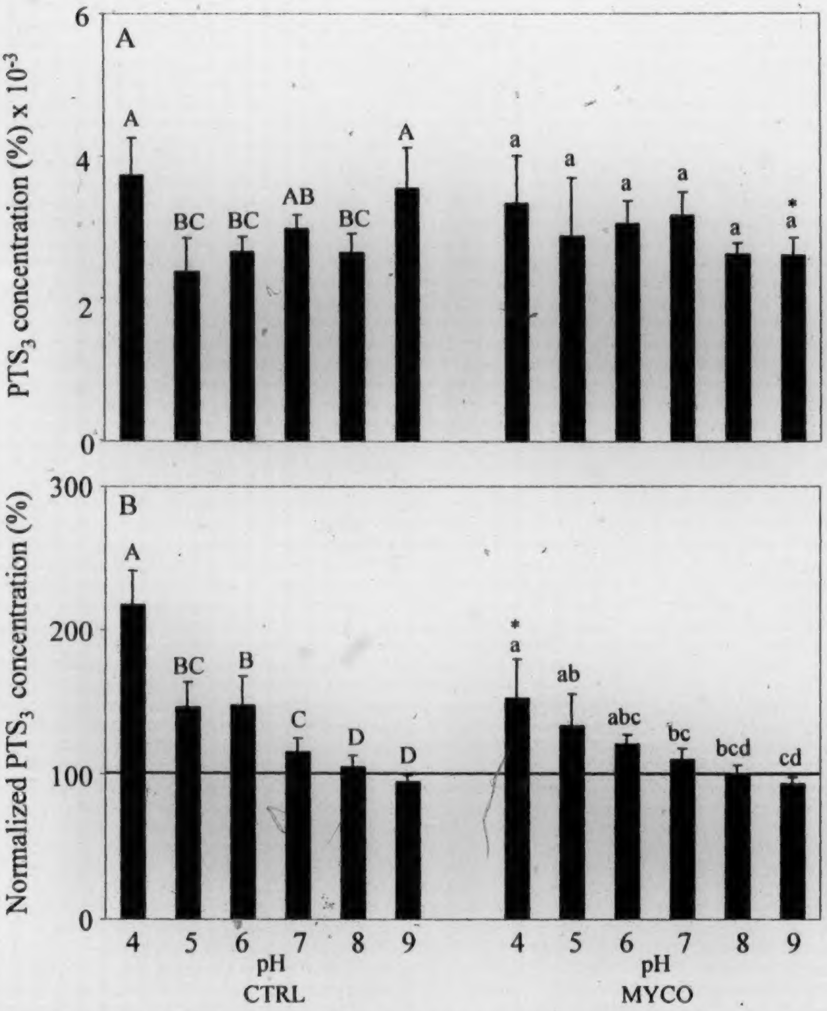

Figure 4.2. (**A**) PTS$_3$ concentration in xylem exudate measured prior to the addition of HgCl$_2$. (**B**) Normalized PTS$_3$ concentration measured after the addition of 100 μM HgCl$_2$ and calculated as a percentage of untreated PTS$_3$ values (shown in **Fig. 4.2A**, and indicated by the line at 100% in **Fig. 4.2B**), in non-mycorrhizal (CTRL) or mycorrhizal (MYCO) aspen seedlings receiving a short-term pH treatment (pH 4-9). Least-squares means + SE are shown (n=10). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among pH treatments within CTRL seedlings), lowercase letters (among pH treatments within MYCO seedlings) and asterisks (between CTRL and MYCO for a given pH treatment).

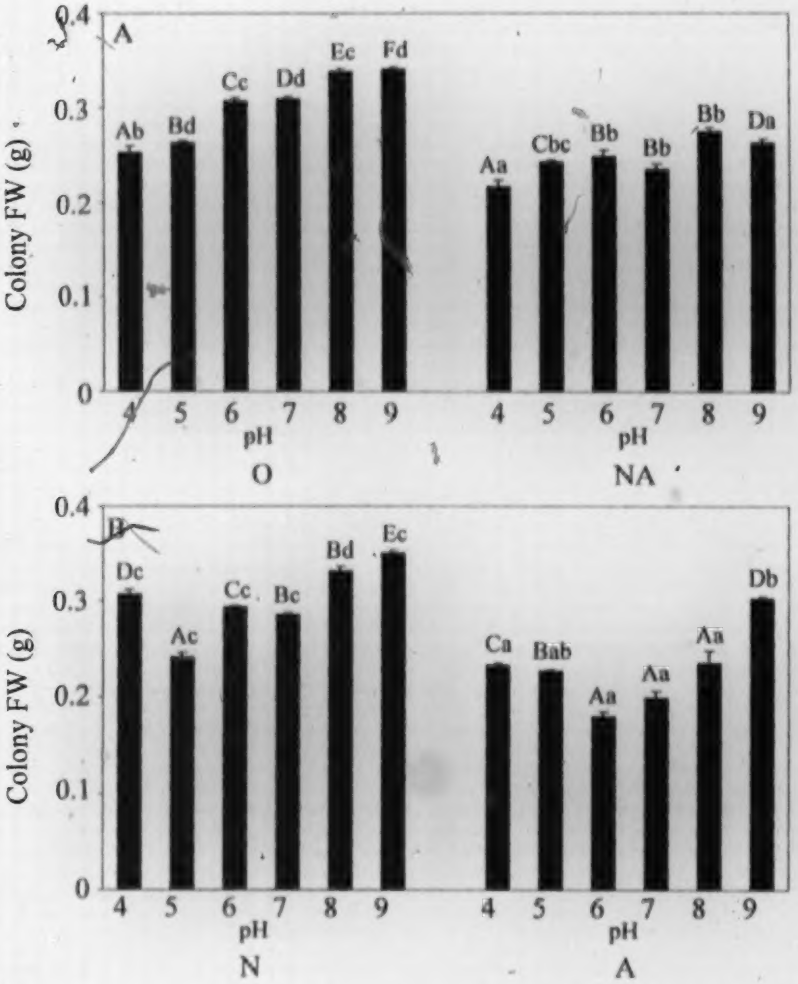

Figure 4.3. Colony fresh weight of *Hebeloma crustuliniforme* pure culture grown on modified MS agar media adjusted to pH treatments of pH 4-9. Nitrogen treatments were no inorganic nitrogen (O) and 8 mM nitrate + ammonium (NA) **(Fig. 4.3A)**, 8 mM nitrate (N) and 8 mM ammonium (A) **(Fig.4.3.B)**. Least-squares means ± SE are shown (n=8). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among pH treatments within O, NA, N, or A treatments separately), and lowercase letters (between O, NA, N, and A treatments for a given pH treatment).

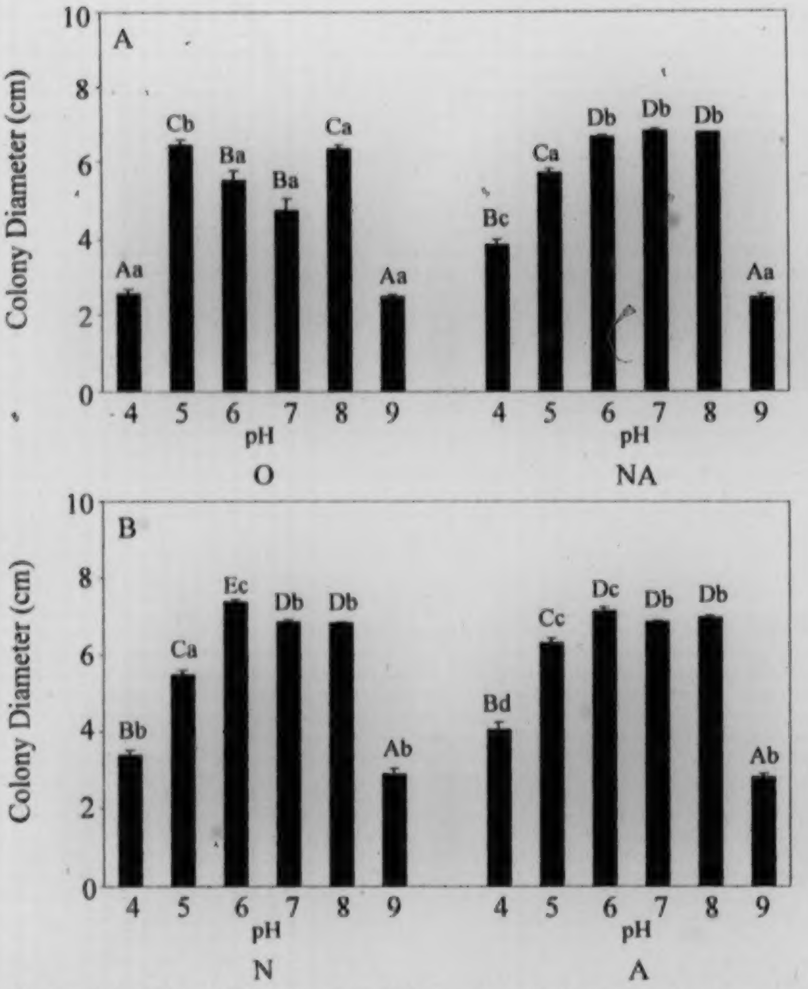

Figure 4.4. Colony diameter of *Hebeloma crustuliniforme* pure culture grown on modified MS agar media adjusted to pH treatments of pH 4-9. Nitrogen treatments were no inorganic nitrogen (O) and 8 mM nitrate + ammonium (NA) (**Fig. 4.4A**), 8 mM nitrate (N) and 8 mM ammonium (A) (**Fig.4.4B**). Least-squares means ± SE are shown (n=8). Significant ($p \leq 0.05$) differences indicated by uppercase letters (among pH treatments within O, NA, N, or A treatments separately), and lowercase letters (between O, NA, N, and A treatments for a given pH treatment).

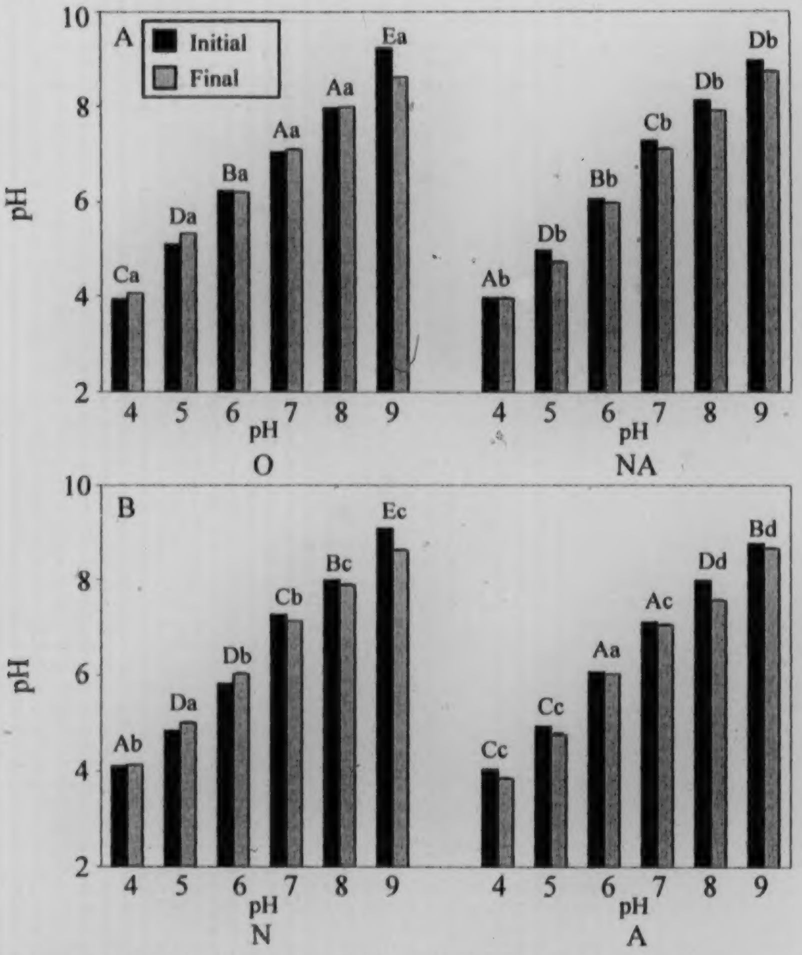

Figure 4.5. Initial pH (prior to addition of *Hebeloma crustuliniforme* mycelial plug to agar treatment plates) and final pH (after growth and harvest of *H. crustuliniforme* colony from agar plates) of MS agar media plates modified to produce different nitrogen treatments, and adjusted to pH treatments of pH 4-9. Nitrogen treatments were no inorganic nitrogen (O) and 8 mM nitrate + ammonium (NA) (**Fig. 4.5A**), 8 mM nitrate (N) and 8 mM ammonium (A) (**Fig. 4.5B**). Least-squares means ± SE are shown (n=8). Significant ($p \leq 0.05$) differences in terms of the change in pH (change = initial – final) are indicated by uppercase letters (among pH treatments within each of the nitrogen treatments), and by lowercase letters (among nitrogen treatments for a given pH treatment).

Table 4.1. ANOVA table indicating p-values of main effects and interactions for growth and pH measurements of *Hebeloma crustuliniforme* in response to nitrogen form (nitrate, ammonium, nitrate+ammonium) and pH (4-9) treatment combinations. Only response variables with at least one significant ($p \leq 0.05$) effect are shown. Effects were tested for significance at $\alpha=0.05$ using PROC MIXED (SAS 9.1). The 'slice=nitrogen form' operation for all response variables indicated significant ($p<0.0001$) differences among pH treatments within each separate nitrogen treatment (no inorganic nitrogen, 8 mM nitrate, 8 mM ammonium, 8 mM ammonium+nitrate).

	Response Variables		
Effects	Colony Diameter	Colony FW	Mean pH
nitrogen	$p<0.0001$	$p<0.0001$	$p<0.0001$
pH	$p<0.0001$	$p<0.0001$	$p<0.0001$
nitrogen * pH	$p<0.0001$	$p<0.0001$	$p<0.0001$

CHAPTER V

Changes in root water relations and root anatomy of ectomycorrhizal and ectendomycorrhizal balsam poplar.

5.1. INTRODUCTION

There are differing views concerning potential benefits of mycorrhizal symbiosis for plants that are exposed to drought and other environmental stresses. Ectomycorrhizal (ECM) fungi have been shown to improve woody plant growth and physiological function, including root water uptake and mitigation of the effects of water deficit stress in a number of studies (Davies et al. 1996, Morte et al. 2001, Landhäusser et al. 2002, Marjanović et al. 2005). These effects may be related to mycorrhizal-induced expression of aquaporins which facilitate root water transport in colonized root systems (Marjanović et al. 2005). However, other studies have also shown no effect of mycorrhizal fungi on root water relations or on growth and productivity in general (reviewed in Jones and Smith 2004). In some cases, certain mycorrhizal species have increased mortality of their host plants or resulted in leaf chlorosis and other colour changes to leaves (Cripps 2001b). Despite this, inoculation of trees and plants with mycorrhizal fungi prior to outplanting has become routine procedure for some nurseries and forestry management operations (Marx and Artman 1979, Malajczuk et al. 1994, Cordell et al. 2000). It has been proposed that aspen may also benefit from pre-inoculation with mycorrhizae (Cripps and Miller 1995). Several factors may affect the extent to which tree growth may visibly and measurably benefit from a mycorrhizal association, including the specificity of plant and mycorrhizal associations (Molina et al. 1992a); the age, health and nutritional status of the host tree species at the time of inoculation (Corrêa et al. 2006); and the particular environmental conditions present during the study (Johnson et al. 1997, Zhou and Sharik 1997). These multiple factors may potentially affect the extent of plant-derived benefits due to mycorrhizal symbiosis, but the difficulty of trying to isolate and control for these variables in mycorrhizal research, particularly field studies, tends to complicate the interpretation of results. The ECM fungus *Inocybe lacera* was shown to improve aspen growth in its natural environment, but became pathogenic when high nutrient concentrations were supplied to the plant (Cripps 2001a,b). This indicates the complexity of plant-mycorrhizal symbiotic interactions, particularly when attempting to compare field with greenhouse studies.

Most boreal forest trees form only ECM associations, although in some tree genera such as *Acer* and *Alnus*, as well as in several families such as *Salicaceae* and *Ulmaceae,* vesicular-arbuscular mycorrhizal (VAM, AM) associations can also form depending upon environmental and other conditions (Smith and Read 1997, Brundrett 2002). Additionally, the *Pinus* and *Larix* genera are known to form ectendomycorrhizal (EEM) associations with E-strain fungal species such as *Wilcoxina* spp.. These fungi have been known to form either EEM or ECM structures with host trees depending upon the tree species. It has been suggested that tree species that do not belong to one of these two coniferous genera may be capable of forming EEM associations (Peterson et al. 2004), especially since EEM fungi are thought to have a broad global distribution with a wide range of plant hosts (Molina et al. 1992a,b). Weakly pathogenic fungal associations were previously misinterpreted as EEM associations, largely indicated and characterized by the presence of intracellular hyphae, although no definitive evidence of EEM associations in deciduous tree species has been experimentally observed to date with current standards for defining true EEM associations (reviewed in Trevor et al. 2001).

Hebeloma crustuliniforme, an ECM fungus, is widely associated with several boreal forest tree species (Kernaghan and Currah 1998), including several members of the Salicaceae family such as *Populus* spp. (Aanen et al. 2000a), as well as species of *Betula*, *Tilia* (Aanen et al. 2000b), *Quercus* (Lunt and Hedger 2003), and *Picea* (Muhsin and Zwiazek 2002b). In previous studies, *H. crustuliniforme* has been shown to increase root hydraulic conductivity (Muhsin and Zwiazek 2002a,b), but has also been shown to have no effect on root hydraulic conductivity (Coleman et al. 1990). Additionally, it is thought that different types of mycorrhizal associations (i.e., VAM vs. ECM) may result in different effects on root water relations in the same plant, which has been experimentally verified in one study (Coleman et al. 1990). Due to the conflicting evidence that mycorrhizal fungi may improve root water relations of host tree species, which may be partly related to the type of mycorrhizal association involved, a number of experiments were conducted to examine these two factors with respect to root anatomy and physiology. The objectives of the present study were: 1) to examine the types of mycorrhizal associations that balsam poplar (*Populus balsamifera* L. subsp. *balsamifera*) formed in association with *Wilcoxina mikolae* var. *mikolae*, and; 2) to examine the effects of mycorrhizal associations with *W. mikolae* var. *mikolae* and *Hebeloma crustuliniforme* on water relations in balsam poplar. I predicted that *H. crustuliniforme* and *W. mikolae* var. *mikolae* would form ECM and EEM associations, respectively, with balsam poplar roots, and that the extent of root colonization as well as the type of mycorrhizal association would affect seedling growth and root water flow properties.

5.2. MATERIALS AND METHODS

5.2.1. Root anatomical characteristics of balsam poplar associated with Hebeloma crustuliniforme and Wilcoxina mikolae var. mikolae

5.2.1.1. Mycorrhizal culture

Hebeloma crustuliniforme (Bull.) Quél. (University of Alberta, Devonian Microfungus Collection, UAMH 5247) and *Wilcoxina mikolae* var. *mikolae* (Yang & Wilcox) Yang & Korf (University of Alberta, Devonian Microfungus Collection, UAMH 6703) were sub-cultured on Melin Norkrans Media (MNM) agar (Mason 1980). MNM-grown fungi were cultured in MNM liquid media and placed on an orbital shaker in aerated flasks for 4 weeks. Mycelia grown in liquid culture was homogenized under sterile conditions using a blender to produce liquid inoculum for seedling roots.

5.2.1.2. Plant culture

Balsam poplar seeds were collected from the North Saskatchewan river valley in Edmonton (Alberta, Canada) in 2006 for immediate use. Seeds were surface-sterilized in 5% sodium hypochlorite for five minutes, then thoroughly rinsed with deionized water, and germinated in sterile, washed silica sand placed in Petri dishes for a minimum of four days. Germinants were then transferred to styroblocks™ (superblock 160/60, Beaver Plastics Ltd., Edmonton, AB, Canada) containing washed, sterile silica sand. Trays were placed in a controlled-environment growth chamber (60% RH, 18 hr photoperiod, 22°C/18°C day/night, 300 μmol m^{-2} s^{-1} PAR with full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada). Trays were bottom-watered every other day with deionized water for four weeks.

5.2.1.3. Mycorrhizal inoculation

Randomly selected seedlings were removed from the styroblocks, roots were rinsed free of sand, and aseptically transferred in a sterile laminar flowhood to double Magenta™ tissue culture vessels (V8505, Sigma-Aldrich) attached with Magenta™ couplers (C0667, Sigma-Aldrich) containing washed, sterile, fine silica sand. During transplantation, seedling roots were surface-inoculated with 5 mL homogenized mycelia per seedling root using a wide-tip sterile pipette. Half of the seedlings received *H. crustuliniforme* homogenized mycelia, and most of the remaining seedlings were inoculated with *W. mikolae* var. *mikolae* homogenized mycelia. A minimum of eight seedlings were left without inoculation to serve as controls for comparison.

Seedlings were grown in sealed Magenta™ vessels in the growth chamber. At the 4-, 8-, and 12-week periods following transplantation, a small amount of fertilizer (25 mL 0.05% 20-20-20) was added to the containers. Magenta™ vessels were slightly vented on a periodic basis to ensure adequate aeration, but precautions were taken to prevent possible contamination from air-borne fungi and bacteria.

Seedlings were grown for a total of 16 weeks after germination prior to microscopic examination of roots.

5.2.1.4. Root harvesting and preparation for microscopy

Randomly selected mycorrhizal seedlings were removed from Magenta™ vessels and gently rinsed of sand. Several root segments that appeared to be mycorrhizal were selected for harvesting, based upon their darker root colouration, as well as their shorter, thicker appearance (Brundrett et al. 1996), compared to roots from non-mycorrhizal root systems. Sections up to 0.5 cm from root tips were removed from these selected roots with a razor, and fixed for five days in 2.5% glutaraldehyde in 0.1 M HEPES buffer, pH

6.8. The methods for root fixation, embedding, sectioning, and microscopy were carried out as described by Melville et al. (1998). Root sections were serially dehydrated in ethanol and embedded in LR-White resin. Several cross-sections were made of the resin blocks with a glass-knife ultramicrotome along the lengths of the root segments, and prepared for laser scanning confocal microscopy. Sections were stained for 90 seconds with 0.5% Sulforhodamine G, a fluorochrome that provides even staining of cell and fungal anatomical components for clear visualization of structures with minimal background fluorescence of LR-white resin (Melville et al. 1998). Permount was used to preserve the sections.

5.2.2. Effects of mycorrhizae on water relations of balsam poplar

5.2.2.1. Plant culture

Populus balsamifera L. subsp. *balsamifera* plants were grown from cuttings taken from the previous year's growth of 7-10 year old balsam poplar trees (minimum 20 trees sampled) collected in Edmonton, Alberta, Canada, in late winter just prior to bud-break (March, 2005). Cuttings 10-15 cm in length, each with at least one dormant bud, were stored wrapped in plastic in cold storage (4°C) in the dark until ready for use.

Prior to use, cuttings were removed from cold storage, rinsed with 75% ethanol solution for up to 1 minute at room temperature, then rinsed with deionized water and soaked in sterile deionized water in trays at room temperature for up to 3 days. Cuttings were then inserted up to the middle of the uppermost bud into 10 cm pots lined with cheesecloth and filled with sterile, washed coarse sand to ensure adequate drainage. Pots were arranged randomly in a controlled-environment growth chamber. Growth chamber conditions were 60% RH, 18 hr photoperiod, 22°C/18°C day/night, 300 μmol m^{-2} s^{-1} PAR with full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada). Most cuttings exhibited bud-break and leaf growth within 2 weeks following placement into the growth chamber. Cuttings that did not flush were removed from the experiment. Cuttings were top-watered every other day with deionized water.

5.2.2.2.. Mycorrhizal treatment

Mycorrhizal inoculum was prepared as described above (section 5.2.1.1.). Four weeks after transferring cuttings to the growth chamber, pots were placed into shallow, lined trays. One-quarter of the cuttings were inoculated with *H. crustuliniforme* (Hc treatment) by injecting a total of 5 mL of homogenized mycelia with a wide-tip sterile pipette into the sand at 2-3 locations closely surrounding the stem, and at a minimum of 3 cm below the surface of the sand. One-quarter of the cuttings were inoculated with *Wilcoxina mikolae* var. *mikolae* (Wm treatment) in the same manner. The remaining plants were separated into two separate control groups to serve as non-mycorrhizal controls for the two mycorrhizal treatments. The control cuttings were inoculated with 5 mL autoclaved, fungal-free MNM liquid media in the same manner as for the mycorrhizal seedlings.

Groups of mycorrhizal-treated and non-mycorrhizal plants were kept physically separated from each other in the same growth chamber, to prevent contamination of non-mycorrhizal plants with fungal inoculum introduced to mycorrhizal plants, during periodic rearrangement of pots and during watering. Plants were top-watered every other day with a small quantity of deionized water for two weeks following inoculation. For the third week following inoculation, plants were bottom-watered with deionized water every other day. Beginning on the fourth week following inoculation, plants were bottom-watered with 0.05% 20-20-20 fertilizer solution once per week in addition to their regular watering schedule.

Plants were grown for twelve weeks following inoculation to ensure adequate colonization prior to measurements.

5.2.2.3. Measurements

5.2.2.3.1. Stomatal conductance and shoot water potential

Stomatal conductance (g_s) was measured *in situ* for intact plants within the growth chamber using an LI-1600 steady-state porometer (LI-COR, Lincoln, NE, USA), and a 2 cm^2 aperture setting with the broadleaf attachment. Leaves with minimal or no necrosis at approximately the mid-region of the stems were selected for measurements.

Shoot water potential (Ψ_w) measurements were conducted using a Scholander pressure chamber, as previously described (Siemens and Zwiazek 2003). The entire shoot, from the point of origin from the original cutting to the shoot tip, was used.

5.2.2.3.2. Shoot height, cutting diameter, and leaf area

Shoot height measurements were taken in intact plants, from the base of the stem to the shoot tip. Diameter measurements of branch cuttings were measured with calipers at the location on the stem just above the surface of the sand in which the cutting was grown.

Projected leaf area measurements were recorded for all the leaves from each plant by removing the leaves and measuring their total surface area with an LI-3100C leaf area meter (LI-COR, Lincoln, NE, USA).

5.2.2.3.3. Root volume and root hydraulic conductivity

Three days prior to harvesting, water-tight, pressure-resistant silicone sealant was applied to the upper surface of the cutting to ensure that no water would flow through the top of the original cutting during root pressurization.

To measure root hydraulic conductivity, half of the plants from each treatment were detopped so that approximately 6-8 cm of the shoot originating from the cutting remained attached to the cutting and to the root system. Roots were placed in Scholander pressure chambers (PMS Instruments, Corvallis, OR) in deionized water. The water was continuously aerated with a magnetic stir bar using a magnetic stirrer placed underneath the pressure chamber. Root water flow rate (Q_v, $m^3\ s^{-1}$) was measured for a minimum of 20 minutes at hydrostatic pressures of 0.3, 0.6, and 0.9 MPa, a range within which changes in Q_v are known to be linear with pressure changes (Siemens and Zwiazek 2003, 2004). A minimum interval of 10 minutes was maintained between Q_v measurements at each increasing pressure to stabilize Q_v values. Once initial root water flux (J_v, $m^3\ s^{-1}\ MPa^{-1}$) measurements were made, values were divided by root volume to obtain root hydraulic conductivity (L_{pr}, $s^{-1}\ MPa^{-1}$)

Root volumes were measured for each root system using volume displacement of water in a graduated cylinder (Voicu and Zwiazek 2004).

5.2.2.3.4. Activation energy and PTS₃ concentrations in xylem exudate

Activation energy of root water flux (E_a, kcal mol^{-1}) was measured using Scholander pressure chambers (Wan and Zwiazek 1999) for roots detopped in the same manner as described above (section 5.2.2.3.3.). Roots were immersed in an aqueous solution of 0.02% trisodium 3-hydroxy-5,8,10-pyrenetrisulfonic acid (PTS_3), a fluorescent tracer dye restricted to the apoplastic pathway of water movement (discussed by Steudle and Peterson 1998). Previously, PTS_3 has been used to measure relative changes in water transport through the apoplast (Siemens and Zwiazek 2003, Schaider et al. 2006). The solution was continuously aerated with a magnetic stir bar using a magnetic stirrer placed underneath each pressure chamber. A circulating water bath (Haake C, F3 digital control, Thermo Electron Corp., W. Germany) connected to hollow copper coils inserted into the root bathing solution was used to adjust the temperature of the solution inside the pressure chambers by cooling the copper coils immersed in the root bathing solution. A digital thermometer probe was inserted into one of the pressure chambers to monitor actual temperature changes of the root bathing solution. Q_v measurements were collected at constant 0.3 MPa hydrostatic pressure for a minimum of 20 minutes at each decreasing temperature (25, 20, 15, 10, and 5°C), with a minimum of 15 minutes wait time between Q_v measurements at each temperature for stabilization of Q_v values. Activation energy was calculated from Arrhenius plots of the slope of $\ln(L_{pr})$ and temperature $(°K)^{-1}$ using the formula:

$$Ea = -[R \times \ln K_2 - \ln K_1)/(T_2^{-1} - T_1^{-1})]$$

where $R = 1.987 \times 10\text{-}3$ kcal mol^{-1} T^{-1}

$K_2 = L_{pr}$ at point 2

$K_1 = L_{pr}$ at point 1

T_2 = temperature (°K) at point 2

T_1 = temperature (°K) at point 1

The designations "point 2" and "point 1" in the equation refer to the end-point locations on the Arrhenius plots between which there was a linear relationship.

Xylem sap samples were collected from pressurized detopped roots following each set of Q_v measurements, at each of the five temperatures, for measurement of xylem exudate PTS_3 concentrations. Samples were diluted with water and measured for PTS_3 concentration against a PTS_3 standard curve using a Sequoia-Turner 450 spectrofluorometer (Apple Scientific, Chesterland, OH, USA) with a 405 nm excitation and 515 nm emission spectrum (Skinner and Radin 1994). PTS_3 concentrations for each root system were plotted over temperature changes to give a value of relative change in PTS_3 concentration per degree Celsius for each seedling.

5.2.2.3.5. Root colonization

Whole root systems were gently washed and preserved in 4:3:3 ethanol-glycerol-water solution, and stored at 4°C in the dark for mycorrhizal analysis. A sub-sample of roots from each treatment was selected for root colonization measurements. Root systems were removed from preserving solution and dissected to collect all fine roots with a diameter of $\leq$0.05 mm. These fine roots were cut into 1-2 cm length pieces, randomly mixed, and spread out in a large, water-filled Petri dish. Approximately one-quarter of the fine roots were removed from the batch and stored in sealed tubes filled with preserving solution.

Staining of mycorrhizal roots was conducted similarly to the procedure outlined by Brundrett et al. (1996). Up to half of the tube-stored roots were removed, rinsed well with deionized water on a mesh filter, and root surfaces were blotted with tissue paper. Root samples were placed in glass scintillation vials filled up to 1/3 volume with 10% KOH and autoclaved for 20 minutes. Roots were then removed from KOH solution, rinsed well with deionized water on a mesh filter, and excess water was removed from root surfaces with tissue paper. Root samples were placed in scintillation vials containing 0.05% trypan blue-lactoglycerol staining solution, and autoclaved for 12 minutes. When cool, trypan blue solution was removed from the scintillation vials, and roots were washed several times first with deionized water, and then with lactoglycerol to remove excess stain. Stained roots were then left in lactoglycerol for a minimum of five days to ensure adequate destaining of roots.

Destained roots were spread out in lactoglycerol solution in plastic Petri dishes marked with a 4 x 4 – 1 cm^2 grid. The counting of dark- and light-stained root segments intersecting the horizontal and vertical lines of the grid was conducted to determine percent root length colonization (Brundrett et al. 1996).

5.2.2.4. Statistical analysis

All data were analyzed with SAS 9.1. (SAS Institute, North Carolina, USA) to determine statistically significant ($p \leq 0.05$) differences between mycorrhizal treatments All data were first tested for normality of distribution and homogeneity of variance. Residuals from the data sets were calculated and used to graph normal probability plots and box plots, to determine if there were any statistical outlier values in the data sets. Outliers, if any, were removed from the data sets.

Measurements (as described in section 5.2.2.3.) were analyzed using a single-factor MIXED ANOVA to compare the two mycorrhizal (Hc or Wm) treatments with each other and with the non-mycorrhizal control (CTRL) group. A block design was used for analysis because harvesting of seedlings occurred over several days, with at least one seedling from each treatment group harvested per day. Therefore, the days of measurements were the blocks.

The statistical model used for the statistical analysis was:

$Y_{ijk} = u + M_i + B_j + e_{ijk}$, where

Y_{ijk} = value of individual observation (*i*=mycorrhiza, *j*=block, *k*=observation)

u = overall mean of observations

M_i = effect of i^{th} treatment (*i* = mycorrhizal treatment)

B_j = effect of j^{th} block (*j*=day of measurement)

e_{ijk} = residual error

Comparisons from the MIXED ANOVA were conducted using least-squares means (α=0.05). The results of all statistical analyses are presented in figures and tables at the end of this chapter.

5.3 RESULTS

5.3.1. Stomatal conductance, shoot height, and leaf area

Least-squares means and standard errors for stomatal conductance (g_s), shoot height, and total leaf area are shown (Fig. 5.1). Both g_s (Fig. 5.1A) and shoot height (Fig. 5.1B) of Wm plants were significantly higher by 30% compared to Hc and non-mycorrhizal control (CTRL) plants (247.4 ± 29.9 mmol m^{-2} s^{-1}, 17.62 ± 1.85 cm) (Fig. 5.1A,B). There were no significant differences in g_s and shoot height between Hc and CTRL plants.

Relative to CTRL plants (132.7 ± 13.75 cm^2), mean leaf area of Wm plants increased, and remained unchanged in Hc plants. Only the differences between Wm and Hc and Wm were significant, with Wm plants having almost twice the leaf area of Hc plants (Fig. 5.1C).

5.3.2. Shoot water potential, cutting diameter, activation energy, and xylem exudate PTS$_3$ concentrations

Unlike shoot height and leaf area measurements, mean diameter of branch cuttings increased with Hc and decreased with Wm plants relative to CTRL (6.89 ± 0.37 mm), although only the differences between Hc and CTRL plants and between Wm and Hc plants were significant. Mean shoot Ψ_w showed the opposite trend as for stem diameter with respect to differences between the three groups, although the differences were not significant (Table 5.1).

Although there were slight differences between E_a and Δ PTS$_3$ values between the three treatment groups, these differences were not significant (Table 5.1).

5.3.3. Root colonization

The least-squares mean percentages of root length colonized by mycorrhizal fungi for treatments and controls are shown (Fig. 5.2A). Both Hc and Wm plants had at least twice the percentage of colonized root length than the CTRL plants (22.34 ± 2.89%), which was a significant increase (Fig. 5.2A). There were no significant differences between Hc and Wm plants.

5.3.4. Root volume and root hydraulic conductivity

Root volume significantly increased with Wm but not with Hc relative to CTRL (5.46 ± 0.50 cm^3) plants (Fig. 5.2B). Differences between Hc and Wm were also significant. In contrast, L_{pr} increased with Hc and Wm relative to CTRL ($2.00 \times 10^{-5} \pm 3.35 \times 10^{-6}$ s^{-1} MPa^{-1}) plants, but only the difference between Hc and CTRL plants was significant (Fig. 5.2C).

5.3.5. Structural anatomy of colonized roots

Laser scanning confocal microscopy images of balsam poplar roots infected with Hc and Wm and stained with sulforhodamine G are shown (Fig. 5.3). Figs. 5.3A and 5.3B show a lower-resolution image of an Hc root cross-section and a close-up of the mycelial structures within that same section, respectively. Figs. 5.3C and 5.3D show a lower-resolution image of a Wm root cross-section and a close-up of part of the mycelial structures within that same section, respectively. In the Hc root, a mantle is clearly visible around the outside of part of the root, which is indicative of an ectomycorrhizal association, but there is no distinct Hartig net visible (Fig. 5.3A,B). In the Wm root, there is a mantle surrounding the outside of the root, and an extensive Hartig net, located between cortical cells, that extends all the way to the vascular cylinder (Fig. 5.3C,D). Some of the cortical cells contain faintly fluorescing segments of structures that are not present in the Hc root, which may be intracellular hyphae and indicative of an ectendomycorrhizal association (Fig. 5.3D).

5.4. DISCUSSION

Hc and Wm produced different mycorrhizal structures with associated balsam poplar roots. In the present study, Hc formed an ECM association with balsam poplar, as indicated by a partial mantle with hyphal projections around sectioned roots, although there was little evidence of a Hartig net around the first layer of cortical cells (Fig. 5.3 A,B). The fact that Hc did not appear to be well associated with balsam poplar roots, given the lack of a full mantle and Hartig net, was surprising due to its known associations with *Populus*. It was expected that Hc would produce a mantle and Hartig net, which are characteristic ECM structures, since Hc is an ECM fungus with a broad host range (Smith and Read 1997) and is known to associate with species of *Populus* (Hutchison 1991).

In contrast, Wm appeared to form characteristic EEM structures (reviewed in Trevor et al. 2001, Peterson et al. 2004), mainly consisting of a full mantle and a Hartig net that extended through the root cortex (Fig. 5.3 C,D). It is also possible that some of the faintly fluorescing structures within some of the cortical cells may have been intracellular hyphae (Fig. 5.3 C,D). However, it was not possible to determine that there were in fact intracellular hyphae. Furthermore, the presence of intracellular hyphae may not be necessary for a true EEM association, since intracellular hyphae may only occur in senescing roots (EEM characteristics reviewed in Trevor et al. 2001, Peterson et al. 2004). Wm is an E-strain fungus capable of forming ECM and EEM structures (Peterson et al. 2004) and could potentially form either an ECM or EEM association with poplar. To the best of my knowledge, the type of association of Wm with poplars had not been previously described.

This may be the first instance reported that E-strain fungi are capable of forming EEM associations with deciduous tree species. Since this association was produced in a controlled growth chamber environment, further studies will be needed to determine whether such associations could also form in balsam poplar trees growing in their natural environments, particularly in the presence of coniferous trees. It is known that different tree species can be connected through mycorrhizal networks of the same fungal species (Simard and Durall 2004), but to the best of my knowledge, the involvement of E-strain fungi in mycorrhizal networks has never been investigated. Although many species of ectomycorrhizal (ECM) fungi have been well characterized, little is known about E-strain fungi and their potential ecological and physiological roles in relation to angiosperm tree species that grow in association with coniferous tree species known to form associations with E-strain fungi. It is not known if E-strain fungi may have physiological effects on plants that are more similar to an ECM or an EEM fungus.

The percent of colonized roots from either treatment was approximately the same (~50 %), and approximately twice of that in non-mycorrhizal (CTRL) roots (Fig. 5.2A). These results illustrate the difficulty of maintaining an aseptic environment in a controlled growth chamber, even though mycorrhizal plants were kept physically separate from CTRL plants. This has been observed by Nardini et al. (2000) who reported 25% infection rate for non-ECM roots compared with almost 100% infection for ECM roots. However, the present results also indicate that any growth or physiological differences between CTRL and mycorrhizal plants, or differences between Hc and Wm treatments, are likely due to the effects of the mycorrhizal fungi treatments, rather than the extent of colonization.

Hc and Wm both affected the growth and physiological parameters (g_s, L_{pr}) measured in balsam poplar plants, although the two fungal species differed in their effect. Wm resulted in significantly greater increases in leaf area, stomatal conductance (g_s), shoot height (Fig. 5.1), and root volume (Fig. 5.2B), compared to Hc, which appeared to have no effect upon these parameters. Wm plants also showed a slight, but non-significant increase in shoot water potential (Ψ_w) compared with Hc plants (Tab. 1). Hc resulted in significantly higher root hydraulic conductivity (L_{pr}), (Fig. 5.2C) and cutting diameter (Tab. 1) compared to the L_{pr} and cutting diameter means of Wm plants. These results are consistent with the previous studies that have shown increased growth, g_s, and root water relations in mycorrhizal plants (Nardini et al. 2000, Landhäusser et al. 2002, Bogeat-Triboulot et al. 2004, Sarjala and Potila 2005). They also corroborate the results of the study which reported that several species of ECM fungi resulted in increases in several growth parameters and nutrient assimilation of a single host plant species, but none of the fungal species used resulted in increases with respect to all measured growth and nutritional parameters (van der Heijden and Kuyper 2003). It is evident from the present results that Hc and Wm have different effects upon balsam poplar, which may be partly due to the type of association formed by Hc (ECM) and Wm (EEM).

The effect of mycorrhizae on plants, or lack of effect, may be partly due to different fungal species (Coleman et al. 1990). This may have been a reason for the observed differences in CTRL, Hc, and Wm plants. However, little is known about biochemical and molecular changes occurring in both mycorrhizal plants and fungi that affect overall plant growth and function. Changes at the cellular level associated with symbiosis, such as expression of genes and transport proteins, have been observed (Lei and Dexheimer 1988, Duplessis et al. 2002, Tagu et al. 2002, Marjanović et al. 2005), although the larger implications of these changes with respect to plant physiological functions are not always well understood. In medic and tomato plants inoculated with certain species of arbuscular mycorrhizal fungi, mycorrhizal plants showed increased labeled P uptake at the cellular level that did not result in increased growth at the whole-plant level (Smith et al. 2003). Additionally, increased plant growth and productivity due to mycorrhizal associations may only be evident at the population or community level rather than at the whole-plant level (reviewed in Jones and Smith 2004). The different levels (molecular, cell, plant, population) at which any potential benefit from mycorrhizae can be observed complicate our understanding of what conditions need to be met, or fungal or plant properties need to be present, for mycorrhizal fungi to provide a measure of improved growth or function to their host plants. The fact that Wm increased shoot growth in balsam poplar without having an impact on root water relations, and vice versa for Hc, indicates that there are likely some underlying biochemical and molecular mechanisms responsible for the differences observed.

The cohesion-tension (CT) theory that describes the upward movement of water through xylem, depends upon a tightly-controlled cause-and-effect relationship between g_s, Ψ_w, and hydraulic conductivity (reviewed in Tyree 1997). In this system, g_s is considered to be a driving force for water flux into roots according to the CT theory, although the differences in L_{pr} between Hc and Wm cannot be explained by the differences in g_s. The overall rate of water flux is determined by the largest resistance to water flux within the whole-plant system. Any change in one of these factors could result in predicted changes in the other factors, based upon the principles of the CT theory and the soil-plant-atmosphere continuum (Sperry et al. 2003). The lack of a correlation between these parameters in both Hc and Wm may be indicative of mycorrhizal-induced changes in plants that altered physiological plant processes such as those involved in plant water relations. Alternatively, mycorrhizal associations may have affected (increased or decreased) the largest resistance to water flux within balsam poplar, These results were consistent with a previous study, where increased L_{pr} in mycorrhizal roots was observed in white spruce and trembling aspen without a corresponding increase in g_s (Landhäusser et al. 2002).

It must also be considered that L_{pr} measurements recorded in this study were representative of the potential maximum L_{pr} that can be reached in the absence of other limiting factors within the whole-plant system. Other studies have also attempted to understand the underlying mechanisms for increased L_{pr} in mycorrhizal plants with limited success. Coleman et al. (1990) theorized that changes in L_{pr} due to ECM fungi were correlated with changes in the root-shoot ratio and fine root P concentrations due to ECM colonization, but these correlations were not enough to explain the large differences in L_{pr}. Muhsin and Zwiazek (2002a) reported increased L_{pr} in mycorrhizal seedlings due to increased apoplastic activity, indirectly determined by increased activation energy (E_a).

In the current study, Wm and Hc plants showed slight, but non-significant changes in both E_a and PTS_3 concentrations in xylem exudate (Tab. 1), which may have contributed to, but cannot fully explain the significant differences in L_{pr} between the two mycorrhizal treatments. E_a, a measure of the amount of energy required for the transport of water across cell membranes, varied between 6.55 – 7.8 kcal mol^{-1}, with a lower value for Hc than for CTRL or Wm plants. Previous studies of cell membrane transport have reported that an E_a of $\leq$6 kcal mol^{-1} is indicative of aquaporin (AQP)-mediated passive water transport through individual membranes, whereas restriction of water movement through AQPs increased E_a values (Maurel 1997, Shütz and Tyerman 1997). However, these studies did not measure E_a for whole plant tissues or root systems, which have multiple pathways of resistance to water flux. It is possible that if non-AQP-mediated pathways, such as the apoplast, become a larger contributor to overall L_{pr}, E_a values may decrease because the apoplastic water flux offers less resistance than AQP-mediated water flux. The fact that Hc plants had a slightly lower E_a value may partly explain why they also had a higher L_{pr}. Nevertheless, the large differences in L_{pr} seen between the mycorrhizal and CTRL plants (Fig. 5.2C) cannot be entirely explained by the slight changes in relative proportions of apoplastic flow (estimated by PTS_3 concentration) or changes in resistance to root water movement (estimated by E_a).

5.4.1. Conclusions

The present study demonstrated that Hc formed an ECM association and Wm formed an association which had EEM characteristics in balsam poplar. The two fungal species had different effect on plants, with Wm resulting in increased shoot growth and g_s, and with Hc resulting in increased L_{pr}. The differences in the effects of Hc and Wm occurred despite similar extents of root colonization by the two fungal species. It can be speculated that the different effects may partly have been due to the differences in the structures of ECM (shallow Hartig net) and EEM (extensive Hartig net, possible intracellular hyphae) associations of Hc and Wm, respectively. The different effects may also have been partly due to possible differences in the molecular or cellular changes in balsam poplar or fungal tissue resulting from the symbiotic process. These findings regarding the effects of Wm in balsam poplar have important implications for future research, since balsam poplar may be present in forest stands with *Pinus* and *Larix*. It is possible that E-strain fungi present in boreal soils may form a combination of interconnecting ECM and EEM mycorrhizal networks between coniferous and deciduous tree species, including balsam poplar. Further research is required to better understand the ecological role fulfilled by EEM fungi, particularly in association with angiosperm tree species, and how the biochemical and cellular processes that occur during ECM and EEM symbiosis may affect the host plant.

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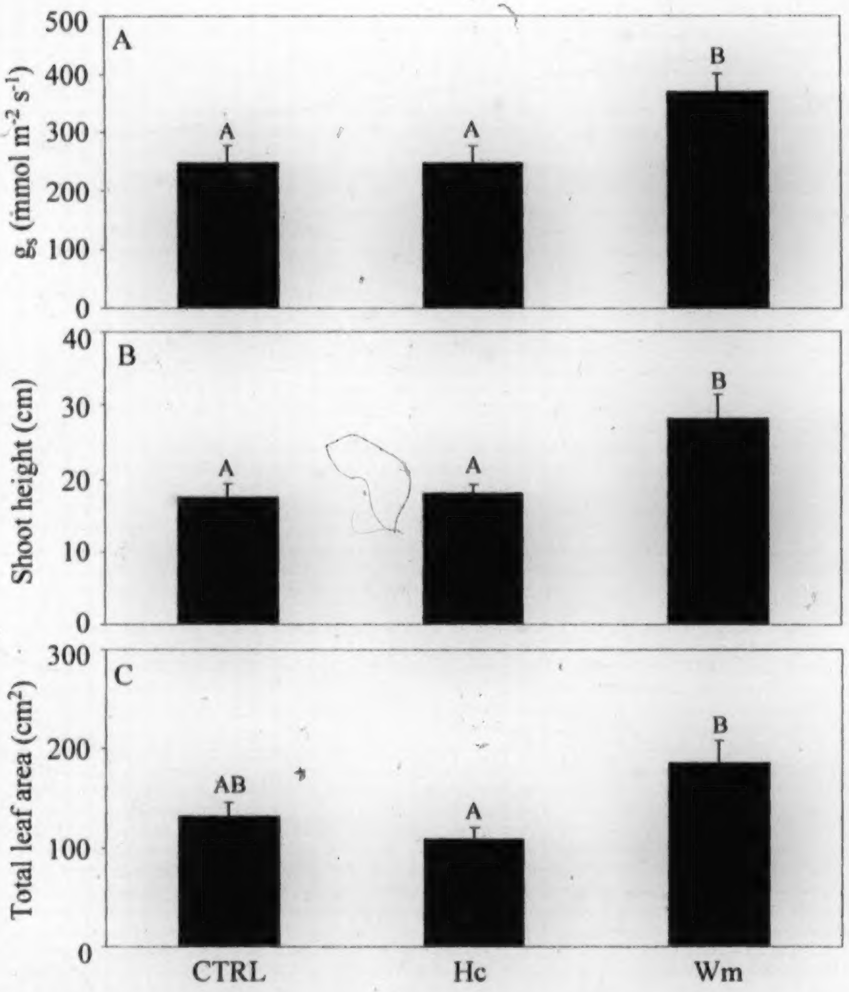

Figure 5.1. (**A**) Stomatal conductance (g_s), (**B**) shoot height, and (**C**) total leaf area, in non-mycorrhizal (CTRL) or mycorrhizal balsam poplar seedlings inoculated with *Hebeloma crustuliniforme* (Hc) or *Wilcoxina mikolae* var. *mikolae* (Wm). Least-squares means ± SE are shown (minimum n=8). Significant ($p \leq 0.05$) differences between treatments indicated by uppercase letters.

Table 5.1. Shoot water potential (Ψ_w), cutting diameter, activation energy (E_a), and slope of change in PTS_3 concentration in xylem exudate with temperature change (Δ PTS_3) in non-mycorrhizal (CTRL) or mycorrhizal balsam poplar seedlings inoculated with *Hebeloma crustuliniforme* (Hc) or *Wilcoxina mikolae* var. *mikolae* (Wm). Least-squares means ± SE are shown (minimum n=8). Significant ($p \leq 0.05$) differences between treatments indicated by different lowercase letters.

Measurements	CTRL	Hc	Wm
Shoot Ψ_w (MPa)	-0.894 ± 0.0512^a	-1.12 ± 0.114^a	-0.807 ± 0.0415^a
Cutting Dia. (mm)	6.89 ± 0.37^a	8.73 ± 0.44^b	5.75 ± 0.37^a
E_a (kcal mol^{-1})	7.20 ± 0.419^a	6.55 ± 0.768^a	7.765 ± 0.900^a
Δ PTS_3 (% °C^{-1})	$-4.88 \times 10^{-5} \pm 1.20 \times 10^{-5a}$	$-3.80 \times 10^{-5} \pm 9.99 \times 10^{-6a}$	$4.07 \times 10^{-5} \pm 7.36 \times 10^{-6a}$

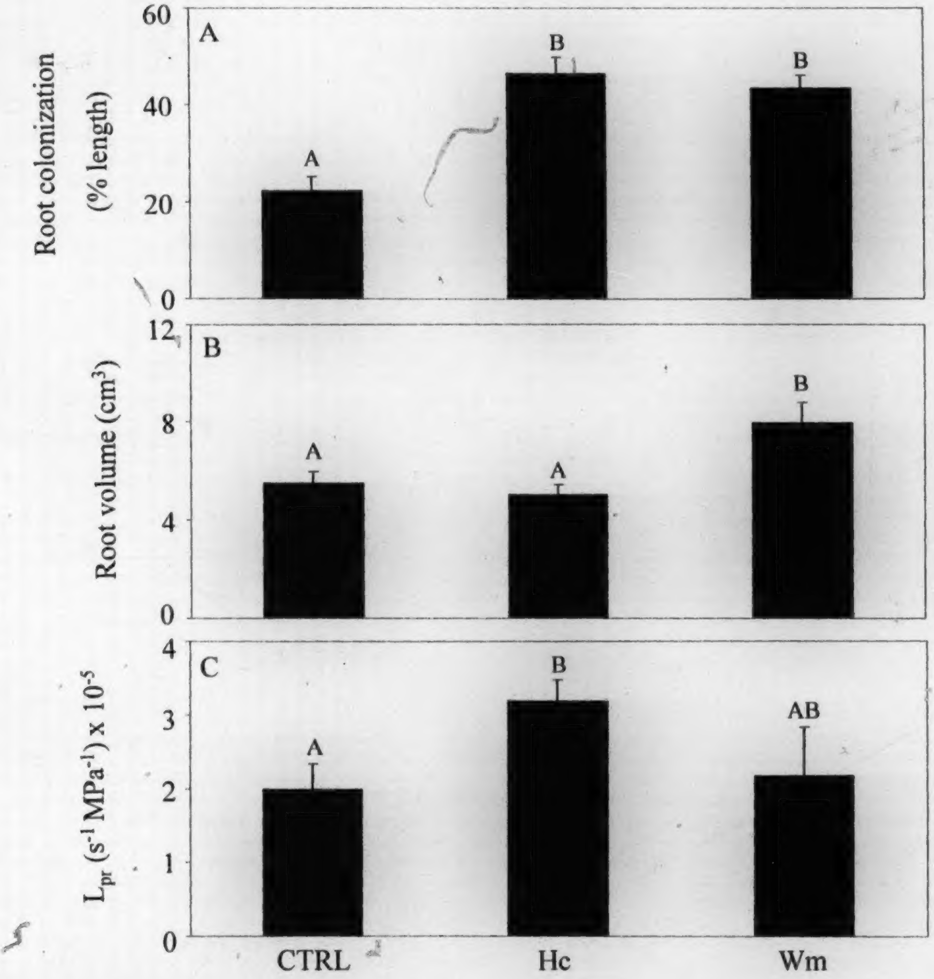

Figure 5.2. (**A**) Root colonization, (**B**) root volume, and (**C**) root hydraulic conductivity (L_{pr}), in non-mycorrhizal (CTRL) or mycorrhizal balsam poplar seedlings inoculated with *Hebeloma crustuliniforme* (Hc) or *Wilcoxina mikolae* var. *mikolae* (Wm). Least-squares means ± SE are shown (minimum n=8). Significant ($p \leq 0.05$) differences between treatments indicated by uppercase letters.

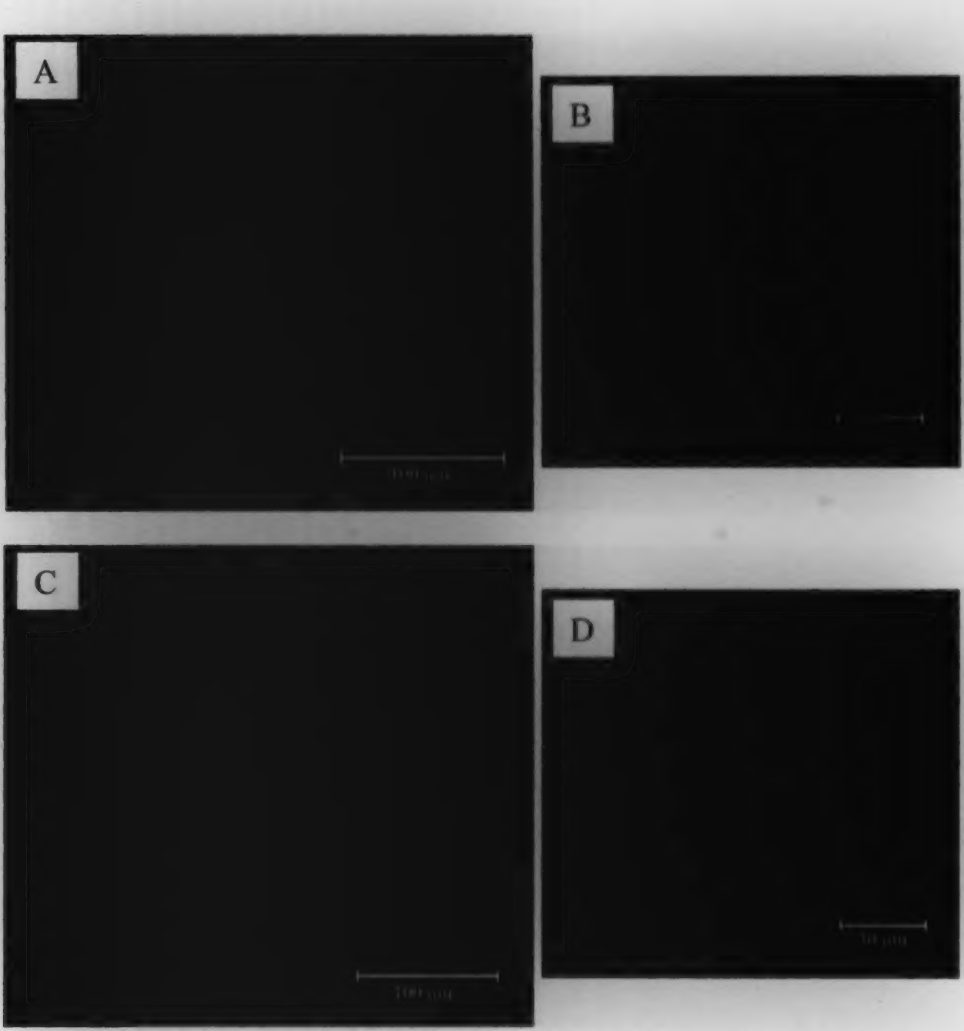

Figure 5.3. Images of 4 month-old balsam poplar seedling roots inoculated with *Hebeloma crustuliniforme* and *Wilcoxina mikolae* var. *mikolae*. (**A**) and (**B**) show root cross-sectional and magnified images of *H. crustuliniforme* roots, respectively. (**C**) and (**D**) show root cross-sectional and magnified images of *W. mikolae* var. *mikolae* roots, respectively. Root segments were embedded in LR-White, sectioned at least 5 mm from root tip, and stained with sulforhodamine G, for examination with laser scanning confocal microscopy.

CHAPTER VI

Synthesis

6.1. General Discussion and Conclusions

The overall objectives of the studies described in Chapters II-V were to add to the current body of knowledge of how mycorrhizal fungi can assist their host plants in improving their growth, water relations, and nitrogen assimilation under different pH conditions and with different inorganic nitrogen fertilization treatments. This research is considered to be important for several reasons:

(1) Although many studies have documented positive effects of ectomycorrhizae on overall growth, productivity, physiology, or fitness of the host plants (Jentschke et al. 1991, Cordell et al. 2000, Jentschke and Godbold 2000, Morte et al. 2001, Plassard et al. 2002, Muhsin and Zwiazek 2002a, b, Marjanović et al. 2005), other reports have failed to find any effects (Bronstein 1994, Johnson et al. 1997, Cripps 2001, Jones and Smith 2004).

(2) The reasons why plants may not always benefit from mycorrhizal symbiosis are complex, and affected by factors such as: age, health, and nutritional status of the plant; biotic, climatic, and edaphic conditions; and species specificity of the plant and fungi involved in the association (Molina et al. 1992, Bronstein 1994, Johnson et al. 1997, Jones et al. 2003, Corrêa et al. 2006).

(3) The cellular and physiological mechanisms by which ectomycorhizae affect plant responses to stresses are not well understood. In particular, the interactions and transfer of substances between plants and fungi at the root-hyphal interface in ectomycorrhizal (ECM) and ectendomycorrhizal (EEM) associations, and the cellular and molecular changes that may occur in both plants and fungi as a result of the process of symbiosis, have not been studied, although there is evidence of such symbiosis-induced functional (Lei and Dexheimer 1988, Marjanović et al. 2005, Uehlein et al. 2007, Frettinger et al. 2007) and structural (Tagu et al. 2002, Duplessis et al. 2002, Bogeat-Triboulot et al. 2004) changes.

6.1.1. Summary of new research presented

6.1.1.1. Completion of study objectives

The new research presented in this dissertation met the four study objectives (outlined in Chapter I). Chapters II and III were consistent with the first objective, which was to assess the long-term effects of different forms and levels of inorganic nitrogen (ammonium and nitrate) treatments on the growth, water relations, nitrogen assimilation, and nitrogen metabolizing activity of trembling aspen with and without mycorrhizal fungi. Chapter III was consistent with the second objective, which was to assess the long-term effects of different forms of nitrogen on the growth and nitrogen metabolizing activity of a pure-culture ectomycorrhizal fungus grown on media at a range of different pH conditions. Chapter IV was consistent with the third objective, which was to examine the effects of the ectomycorrhizal fungus *Hebeloma crustuliniforme* on the water relations of trembling aspen exposed to a range of different pH conditions in the short-term. Chapter V was consistent with the fourth objective, which was to examine the effects of an ectomycorrhizal fungus (*Hebeloma crustuliniforme*) and an E-strain fungus (*Wilcoxina mikolae* var. *mikolae*) on general water relations and root anatomy in balsam poplar.

6.1.1.2. Summary of new research and verification of hypotheses tested

The studies presented in Chapters II and III were designed to address several gaps in our knowledge of mycorrhizal-plant interactions under different nitrogen conditions, and to test hypotheses 1-3 (hypotheses were outlined in section 1.6, Chapter I). Previously, no studies of aspen's ammonium tolerance at the whole-plant level had been reported, although trembling aspen had been classified as an ammonium-sensitive species because of cellular studies carried out at low ammonium concentrations (Min et al. 1998, 1999, 2000, Kronzucker et al. 2003). Chapters II and III disproved hypothesis 1 by demonstrating that aspen was capable of assimilating and utilizing low and moderate concentrations of ammonium when it was supplied as the sole source of nitrogen at higher concentrations than previously reported (Min et al. 1998, 1999, 2000). The results suggested that aspen may have had mechanisms for dealing with ammonium at the whole-plant level that were different than those mechanisms at the cellular level. Moderate ammonium tolerance in aspen had not been previously reported. Chapter III also demonstrated that nitrogen assimilation occurs largely in aspen roots, although nitrate and ammonium assimilation may be partitioned between leaves and roots. *H. crustuliniforme* (Hc) did not increase growth of associated aspen, disproving hypothesis 2, but did enzymatically alter nitrogen assimilation of aspen, proving hypothesis 3. Hc facilitated nitrogen uptake and increased ammonium assimilation at higher concentrations. However, when nitrogen concentrations were low, Hc also resulted in higher mortality, possibly due to the increased nutritional cost to host plants associated with having a fungal symbiont. Although some aspects of nitrogen nutrition and ammonium tolerance (Min et al. 1998, 1999, 2000, Marler et al. 2001, DesRochers et al. 2003, van den Dreissche et al. 2005, Choi et al. 2005), and the effect of nitrogen on water relations (Radin and Matthews 1989, Li and Shao 2003, Guo et al. 2007) had been previously addressed in aspen and hybrid poplar, the influence of mycorrhizal associations on nitrogen nutrition have received little attention.

The studies presented in Chapters IV and V were designed to further address several aspects of the effects of mycorrhizal fungi on root water relations, aquaporin (AQP) activity, and how short-term pH stress may affect aspen and mycorrhizal fungi. Chapter V was also designed to determine if balsam poplar could form an ectendomycorrhizal association with an E-strain fungus. These areas of mycorrhizal research had not been previously studied. Chapters IV and V demonstrated that Hc was capable of increasing root hydraulic conductivity (L_{pr}) under alkaline conditions, proving hypothesis 4. This increase in L_{pr} in Hc-associated aspen may be due to mechanisms that do not involve AQP-mediated root water flux. Chapter IV also demonstrated that, although L_{pr} of non-mycorrhizal aspen was not affected by short-term pH changes, Hc increased aspen's alkaline pH tolerance, possibly as a result of Hc's demonstrated pH preferences, proving hypothesis 5. Additionally, Chapter IV showed that root AQP activity is affected by pH, with an inverse relationship between Hg-sensitive AQP activity and L_{pr}.

The study presented in Chapter V demonstrated that balsam poplar was capable of forming an EEM association with *W. mikolae* var. *mikolae* (Wm), proving hypothesis 7. Additionally, Hc and Wm had different effects on balsam poplar's stomatal conductance (g_s), shoot water potential (Ψ_w), and L_{pr}, partially proving hypothesis 6, which may have been due to the differences in the type of association each fungus formed with balsam poplar. No other study to date has reported a true EEM association involving an angiosperm tree species, using the current criteria for defining an EEM association, although others have suggested that such an association was possible (discussed by Trevor et al. 2001, Peterson et al. 2004).

6.1.2. Summary of Study Results

6.1.2.1. Nitrogen

Trembling aspen has previously been considered to be an ammonium-sensitive tree species, with excess ammonium uptake and accumulation observed at low ammonium concentrations (Min et al. 1998, 1999, 2000). Several other pioneer plant and tree species have also been described as ammonium-sensitive (Britto and Kronzucker 2002, Kronzucker et al. 2003). This was only partly observed in the present studies. In both solution culture and sand culture, trembling aspen generally preferred nitrate, with no evidence of nitrate-induced toxicity at the highest levels of nitrate provided. This was evident in the increased growth, g_s, L_{pr}, and in the nitrate metabolism and ^{15}N uptake studies (Chapters II, III). In solution culture, trembling aspen responded to higher concentrations of ammonium with reduced growth, g_s, and L_{pr} compared to when equivalent concentrations of nitrate were provided (Chapter II). However, at low and moderate ammonium concentrations, trembling aspen showed greater growth, g_s, and L_{pr} than at the same concentrations of nitrate, or in the absence of nitrogen. Although it was not known if potentially toxic effects were present at the cellular level in the presence of moderate concentrations of ammonium, it appeared that aspen required less ammonium than nitrate to support an equivalent level of growth and stomatal and root function (Chapter II). The theory of ammonium toxicity and futile transmembrane cycling in ammonium-sensitive plants (Britto et al. 2001) was partly supported by the slightly higher accumulation of total nitrogen in leaf tissues of ammonium-treated seedlings compared with nitrate-treated seedlings (Chapter II). In sand culture, trembling aspen responded equally well to both nitrate and ammonium, provided that supplied concentrations (8 mM) were sufficient to prevent reduced growth while avoiding mortality as a result of nitrogen deficiency (Chapter III). The fact that sand-grown aspen responded differently than solution-culture-grown aspen could have been due to two factors: 1) differences in root anatomy; and 2) differences in growth history and seed source of seedlings used in these experiments.

Unlike solution-culture-grown aspen, sand-grown aspen roots have an exodermis (Siemens and Zwiazek 2004), which may act as an additional barrier in the restriction of ionic movement through the root apoplast (reviewed in Steudle and Peterson 1998). Additionally, solution culture-grown roots are more likely to have small fissures at the junction of lateral root formations, due to the continuous movement of roots in a circulating aquatic medium, which may result in the unrestricted passage of solutes that can bypass the endodermis and apoplast into the xylem (C.A. Peterson, personal communication). The exodermis may have acted as an additional physical barrier to unregulated ammonium absorption by roots, thereby resulting in less ammonium accumulation in tissues, although this would not have affected any futile transmembrane cycling. Indeed, the total nitrogen concentrations of leaves in the 8 mM nitrate and ammonium solution culture treatments were higher (> 2 %) than in the equivalent treatments for sand-grown aspen (1.4 – 1.5%) (Chapters II, III).

The seedlings used in the sand- and solution-culture studies had different growth histories and origins prior to the experiments. Seedlings used in the solution-culture experiment were one-year-old saplings previously grown in soil from seed collected by the Bonnyville Tree Nursery (Bonnyville, Alberta) and were then hardened for cold storage prior to being flushed in solution culture. Seedlings used in the sand-culture experiment were grown in sand and transplanted into experimental pots at a young age without being hardened and cold-stored. The seeds used for this study were collected from Edmonton, Alberta. The fact that the plants used in solution culture were slightly older, more lignified, and had to adapt to a new growth medium by growing a new root system, may have resulted in some differences in how the older seedlings assimilated the provided nutrient treatments.

There were four findings of interest with respect to aspen's response to inorganic nitrogen.

(1) Even though many of the ammonium and all of the nitrate concentrations used in these experiments were higher than those previously reported in boreal forest soils (Vitousek et al. 1982, Balisky et al. 1995, Jerabkova et al. 2006), aspen was still able to assimilate ammonium and grow when ammonium was the only source of supplied nitrogen (Chapters II, III) and it showed equivalent growth in either 8 mM nitrate or ammonium (Chapter III).

(2) In surviving seedlings, aspen showed greater growth in the presence of 8 mM ammonium vs. nitrate, but mortality was higher for the ammonium-only treatments (Chapter III).

(3) When nitrate and ammonium were provided together, trembling aspen showed reduced growth and nitrogen uptake, but showed higher enzymatic nitrogen assimilation and no mortality. The reduced growth and nitrogen uptake could not be explained by any of the measured parameters, including enzymatic assimilation of nitrate (nitrate reductase) and ammonium (glutamate dehydrogenase), or by lack of changes in pH of sand leachate or xylem exudate (Chapter III). ?

(4) Aspen exhibited slight recovery from ammonium toxicity, indicating that some of the damage caused by toxic effects may be reversible (Chapter II).

These studies suggest that trembling aspen may not be as ammonium-sensitive as previously suggested (Min et al. 1998, 1999, 2000, Kronzucker et al. 2003) and aspen appears to have mechanisms for dealing with ammonium. These mechanisms resulted in good overall growth at the whole-plant level. However, decreased growth due to a combination of nitrate and ammonium could not be explained within the context of these studies. This indicates that the response of aspen to inorganic nitrogen is not straightforward. The utilization of supplied nitrate and ammonium together by aspen merits further investigation at the whole-plant and cellular levels.

6.1.2.2. Mycorrhizal fungi

Mycorrhizal fungi have been known to help improve growth (Bogeat-Triboulot et al. 2004, Sarjala and Potila 2005), root water flux (Landhäusser et al. 2002, Muhsin and Zwiazek 2002a,b), and stomatal conductance (Mason et al. 2000, Nardini et al. 2000) of their plant hosts when they are subjected to environmental stresses. However, several studies have shown that mycorrhizal associations may not confer any benefits to plants (Jones and Smith 2004, Rincon et al. 2006) and can even result in decreased biomass, reduced photosynthesis (Eltrop and Marschner 1996, Corrêa et al. 2006), and low survival (Cripps 2001). Many studies failed to take into account that a mycorrhizal relationship may range from mutualistic to parasitic (Smith and Smith 1996, Johnson et al. 1997), depending upon many factors and their interactions (reviewed in Jones and Smith 2004). This complicates the study of mycorrhizal associations and their effects on host plants. Furthermore, the nature of mycorrhizal associations in angiosperm trees has not been well studied, particularly with respect to symbiotic interactions between the fungus and plant root, cellular and physiological changes induced in both plant and fungus by the process of symbiosis, and the potential ecological and physiological roles of E-strain mycorrhizae that could be associated with angiosperms.

In the present study, it was apparent that *Hebeloma crustuliniforme* (Hc), an ectomycorrhizal (ECM) fungus known to associate with *Populus* spp., and *Wilcoxina mikolae* var. *mikolae* (Wm), an E-strain fungus whose relationship with *Populus* spp. had not been studied, differ from one another in terms of the types of associations formed with balsam poplar and trembling aspen. Hc formed ECM associations with balsam poplar (Chapter V) and trembling aspen (Appendix 1), as expected. However, Hc appeared to form a closer, more well-defined ECM association with aspen than with balsam poplar, given the extent of the mantle and Hartig net structures that were observed in both tree species. In contrast, Wm appeared to form ectendomycorrhizal (EEM) associations with both trembling aspen and balsam poplar (Chapter V, Appendix 1) (R.L. Peterson, personal communication), with the formation of a mantle, a Hartig net through the entire cortex, and the possible formation of unidentified intracellular structures.

It is evident that the two fungal species differed in their effects upon the growth and root water relations of balsam poplar, although it is not possible to state whether these differences were due to the type of association (ECM vs. EEM) between balsam poplar and these two fungi. Wm appeared to increase shoot growth and g_s but not L_{pr}, whereas Hc increased L_{pr} but not shoot growth or g_s (Chapter V). This study illustrated that: 1) different fungi associated with the same plant can result in different benefits to the host plant; and 2) the means by which fungi confer such benefits to the host plant may be biochemical, cellular, or genetic in nature, and not simply due to physical alterations of plant anatomy or structure. According to the cohesion-tension theory, hydraulic movement of water from soil to air is governed by a series of resistances within the plant, and changes in water movement occur as a result of changes in g_s, Ψ_w, and L_{pr} (Tyree 1997, Sperry et al. 2003). However, overall water movement through the plant is still determined by the source of largest resistance within the plant. Wm and Hc had different effects upon g_s, Ψ_w, and L_{pr}, therefore the two fungi may have been affecting areas of major resistance differently (Chapter V). It is possible that cellular or sub-cellular mechanisms were responsible for the changes induced by the fungal symbiosis. However, it was not possible to elicit the reasons for the differences between the two fungi in their effects on balsam poplar in this study.

6.1.2.3. Nitrogen and mycorrhizal fungi

Mycorrhizal fungi have been shown to improve mineral uptake and assimilation in host plants (Smith and Read 1997, Mukerji et al. 2000, Plassard et al. 2002) and alleviate toxicity symptoms with respect to heavy metals and salt (Colpaert and Van Assche 1987, Jentschke and Godbold 2000, Kernaghan et al. 2002). Therefore, it was thought that mycorrhizal fungi associated with trembling aspen may be able to enhance nitrate uptake and to somehow mitigate the toxic effects of ammonium, possibly through enhanced fungal assimilation of ammonium, or through sequestration and utilization of ammonium in fungal tissues. In the present study, no overall growth benefit was apparent at higher nitrogen concentrations (8 mM) (Chapter III). However, mycorrhizal seedlings did have higher total nitrogen and ^{15}N assimilation in leaf and root tissues when either nitrate or ammonium was provided as the only source of nitrogen than when nitrate and ammonium were applied together. (Chapter III).

These results indicate that Hc increased uptake and assimilation of nitrogen, especially ammonium, under conditions of high nitrogen, although increased growth was not a measurable outcome of this improvement in nutrition due to mycorrhizal symbiosis. In spite of the benefits of increased nitrogen assimilation in the presence of higher concentrations of ammonium, there appeared to be a cost to seedlings for having an association with Hc, as indicated by increased mortality under low nitrogen conditions and in the presence of nitrate (Chapter III). Improved ammonium assimilation in mycorrhizal aspen roots was indicated by their lower mortality at high ammonium concentrations, and by lower ammonium concentrations in the xylem exudate of the highest-ammonium treatment, compared with non-mycorrhizal seedlings (Chapter III).

Hc, which preferred alkaline media and nitrate vs. ammonium in pure culture (Chapter IV), appeared to modify the nitrate- and ammonium-assimilating enzymatic functions of its plant host. Hc pure culture was responsive to both the presence of nitrate and ammonium, with increased nitrate reductase (NR) and glutamate dehydrogenase (GDH) activity (Chapter III). Non-mycorrhizal aspen was somewhat more responsive to nitrate (via NR) than to ammonium (via GS or GDH). However, in mycorhizal aspen, NR, GS, and GDH activity was mostly unaffected by form of nitrogen, and NR activity was generally lower in mycorrhizal aspen than in non-mycorrhizal aspen (Chapter III). The results indicated that mycorrhizal aspen was more capable of assimilating ammonium, but was not as capable of assimilating nitrate, compared to non-mycorrhizal aspen. The lack of differences in enzyme activity between the different nitrogen treatments may have been due to the combined responsiveness of aspen to nitrate and of Hc to ammonium in symbiosis (Chapter III).

6.1.2.4. pH and mycorrhizal fungi

Mycorrhizal fungi have been considered for use in land reclamation because of their pH tolerance and because they can increase plant growth and survival in areas of extreme pH (Cordell et al. 1991, Cordell 1997, Wallander 2002, Kernaghan et al. 2002).

Hc showed greater growth in an alkaline environment, which depended upon the form of nitrogen provided, and produced more alkaline-tolerant aspen in this study by increasing L_{pr} and reducing Hg-affected aquaporin (AQP) activity at higher pH (Chapter IV). This study demonstrated that AQP activity can be pH-regulated, consistent with previous studies (Tournaire-Roux et al. 2003, Pettersson et al. 2006), and that maximal AQP activity of aspen is adapted to an acidic environment consistent with aspen ecology. Of particular interest was the fact that maximal L_{pr} coincided with minimal AQP activity, and that initial measurements of apoplastic root water flux and L_{pr} were not well correlated, suggesting that factors other than AQP activity were also influencing overall L_{pr} (Chapter IV). These factors may include water and ion movement through hyphal mycelia and the mantle, and from hyphae to roots, which may provide little resistance to root water flux. Changes in root structure and physiology due to mycorrhizal symbiosis could also have affected L_{pr}. However, properties of mycorrhizal roots are not currently understood, and require further study to better understand the mechanisms by which mycorrhizae may influence the pH tolerance and root water flux of their host plants.

It appeared that the pH tolerance conferred by Hc to aspen may have been partly influenced by Hc's ability to modify the pH of its growth medium. At acidic pH, the extent of Hc's modification was dependent upon the form of inorganic nitrogen present, but at alkaline pH (8-9), Hc was capable of reducing the pH of its growth media by up to 0.6 pH units regardless of the form of inorganic nitrogen provided (Chapter IV). In symbiosis, mycorrhizal aspen also appeared to have lower pH in sand leachate compared with the more alkaline pH values for the leachate of non-mycorrhizal aspen, suggesting that Hc may also have a pH-reducing influence in the soil (Chapter III).

6.2. Suggestions for Future Research

The research objectives of the present study were met, the proposed hypotheses were proven or disproven, and the present study contributed more knowledge to the four areas where more research was required (Chapter I, section 1.6). However, there are several aspects of mycorrhizal symbiosis that merit further research to better understand the relationship between mycorrhizal fungi and angiosperm tree species, and that would enable better interpretation of research results such as those obtained from this study. Areas that would benefit from future research include:

(1) Biochemical, genetic, and structural studies of the changes in both plants and fungi induced by and involved in the process of mycorrhizal symbiosis, complemented by whole-plant physiological studies to determine the functional impact of these changes in both plants and mycorrhizal fungi as organisms. There has been insufficient research in these areas, although it has already been shown that mycorrhizal fungi may directly affect AQP expression or activity in plants (Marjanović et al. 2005, Uehlein et al. 2007).

(2) Metabolic flux studies and functional variability studies for ECM fungi, the need for which have been expressed recently (Koide et al. 2007, Shachar-Hill 2007). Such studies are required to gain a better understanding of fungal-plant-soil relationships as a unit. They could be used to measure and monitor the transfer of water and solutes (i.e., ions, growth substances, minerals, toxic substances) between soil and fungi and between fungi and plants, to examine the roles of various enzymes in fungal tissue, and to monitor the possible assimilation and transformation of nutrients within fungal tissue before transfer to plant roots. Some recent mycorrhizal-host transfer studies have already been conducted with AM fungi (Govindarajulu et al. 2005).

(3) Additional physiological and anatomical studies involving E-strain fungi such as *Wilcoxina spp*, their potential associations with angiosperm tree species, and the possible influence of E-strain fungi on the physiology and ecology of their host trees. Such research has not been conducted or reported thus far (Trevor et al. 2001, Peterson et al. 2004).

(4) pH-regulation of AQPs and root water flux at the cellular and whole-plant level in tree species, similar to the work which has been previously conducted in crop species and at the cellular level (Tournaire-Roux et al. 2003, Alleva at al. 2006, Pettersson et al. 2006).

(5) Water flux studies involving the conductance and regulation of water movement through mycorrhizal structures such as the extraradical hyphae and mantle, as

suggested by Peterson et al. (2004) and Bogeat-Triboulot et al. (2004), and the role of AQPs in mycorrhizal fungi (Frettinger et al. 2007) in the influence of mycorrhizal fungi on root water relations.

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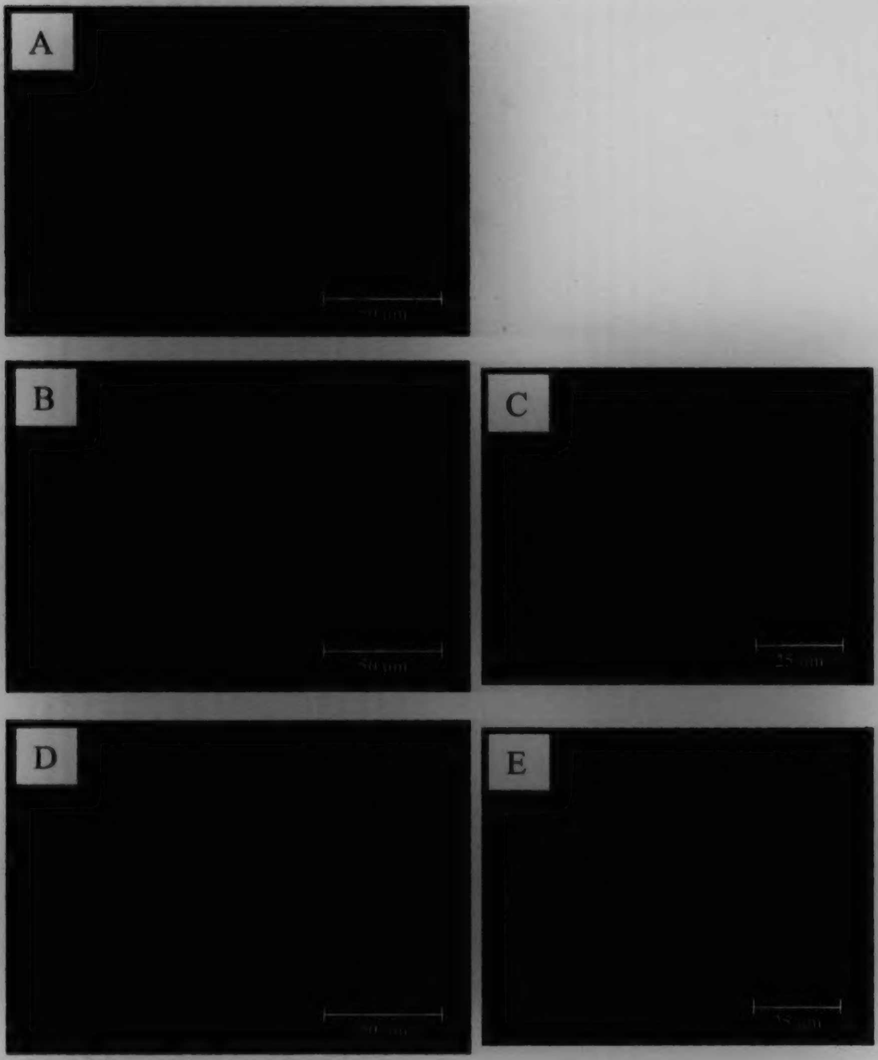Appendix 1. Cross-sectional images of 5 month-old trembling aspen seedling roots that are non-mycorrhizal (**A**), and inoculated with the ectomycorrhizal fungus *Hebeloma crustuliniforme* (**B**, **C**), and the E-strain fungus *Wilcoxina mikolae* var. *mikolae* (**D**, **E**). (**C**) and (**E**) are magnified sections of the images shown in (B) and (D), respectively. Root segments were embedded in LR-White, sectioned at least 5 mm from root tip, and stained with sulforhodamine G, for examination with laser scanning confocal microscopy.